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Pathogenic effects of *Mycoplasma hyopneumoniae* on porcine tracheal organ cultures and neutrophils in vitro, and porcine respiratory goblet cells in vivo

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Iowa State University, 1992

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**Pathogenic effects of Mycoplasma hyopneumoniae on
porcine tracheal organ cultures and neutrophils in vitro,
and porcine respiratory goblet cells in vivo**

by

Mary C. DeBey

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Microbiology, Immunology
and Preventive Medicine
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**Iowa State University
Ames, Iowa**

1992

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GENERAL INTRODUCTION

Mycoplasma hyopneumoniae causes enzootic pneumonia in swine (Mare and Switzer, 1965; Goodwin et al., 1965), the only natural host of the organism. After natural infection, the organism colonizes airway epithelium without invading host tissues.

Despite the fact that distribution of M. hyopneumoniae is limited to airway epithelium, a dramatic host response leading to consolidation of lung lobules is elicited. Mucopurulent exudate is observed in airways, but the severity of this response varies among pigs (Livingston et al., 1972; Ross, 1986).

Mycoplasma hyopneumoniae probably utilizes a number of methods to colonize and survive in the pig. Direct damage to respiratory epithelial cells resulting in ciliostasis and desquamation of epithelium is suggested as a mechanism of pathogenicity for some respiratory mycoplasmas (Cassell et al., 1985), and apparently occurs after infection of pigs with M. hyopneumoniae.

Modulation of the host response appears to be associated with respiratory mycoplasmal infections. Peribronchial, peribronchiolar and perivascular lymphoid infiltration and proliferation, as well as macrophage and neutrophil accumulation suggest that host response to infection contributes to lesion development in mycoplasmal pneumonia

(Cassell et al., 1985).

Mechanisms of pathogenicity of M. hyopneumoniae for cells present in the respiratory tract during infection are not well understood. To undertake a study of pathogenic mechanisms three approaches were taken: 1) histochemical changes in respiratory goblet cell mucin and morphological changes in respiratory tract epithelium were evaluated by image analysis and light microscopy, respectively, after infection of pigs with M. hyopneumoniae; 2) a porcine tracheal ring model to measure ciliostasis and loss of cilia induced by M. hyopneumoniae was developed; and 3) the effect of M. hyopneumoniae on cytoplasmic calcium flux in porcine neutrophils was evaluated in vitro.

Explanation of dissertation format. This dissertation is presented in the alternate format. It contains a general introduction, review of the literature, three manuscripts, general discussion, and appendices. The appendices contain data not submitted for publication in the manuscripts. References cited in the introduction, literature review, and general discussion are listed following the general discussion. Manuscripts were written according to the guidelines for authors of the appropriate journal to which submission was intended. References cited for each manuscript follow the corresponding manuscript. Investigations presented in the manuscripts and appendices

were planned and executed, and the manuscripts written, primarily by the Ph.D. candidate, Mary C. DeBey, with the advice of the major professor, Dr. Richard F. Ross, and other co-authors of the manuscripts.

LITERATURE REVIEW

Disruption of normal function of the mucociliary escalator is evidenced by mucopurulent exudate observed in the airways of pigs infected with Mycoplasma hyopneumoniae. On microscopic examination clumping and loss of cilia are evident in affected airways, and purulent exudate contained in airways is composed primarily of neutrophils and mononuclear cells (Livingston et al., 1972). Alterations of airway mucous gland secretions have been observed after experimental inoculation of swine with porcine mycoplasma (Jones et al., 1975).

Perivascular, peribronchial and peribronchiolar mononuclear infiltration is evident as early as 1 week after experimental infection (Livingston et al., 1972). As the disease progresses alveoli contain edema fluid, neutrophils, and mononuclear and septal cells. During lesion resolution edema fluid disappears leaving collapsed alveoli, and neutrophils disappear from airways (Ross, 1986).

Electron microscopic examination of lung tissue from swine infected with M. hyopneumoniae has revealed that there is close association of the organism with cilia from the epithelial cells of the trachea and bronchi (Mebus and Underdahl, 1977; Underdahl et al., 1980; Tajima and Yagihashi, 1982). Electron microscopy (Tajima and Yagihashi, 1982) and immunofluorescence specific for M. hyopneumoniae

(Amanfu et al., 1984) demonstrated the presence of organisms or antigen on the luminal surface of bronchiolar epithelium. However, immunofluorescence specific for M. hyopneumoniae is not observed in alveoli.

Mycoplasma hyopneumoniae is an extracellular epithelial cell parasite cytotoxic to respiratory epithelial cells. These characteristics, which are common among respiratory mycoplasmas, might imply that epithelial desquamation observed in infected airways is induced by a potent soluble toxin (Gabridge et al., 1985). However, many mycoplasmas apparently induce morphologic and metabolic alterations in host cells through subtle chemical processes rather than by means of a potent toxin (Gabridge et al., 1985).

Indirect evidence of production of a toxin by M. hyopneumoniae is provided by cell damage, visible microscopically, which is induced in airway epithelium in response to infection with M. hyopneumoniae. Direct evidence of a toxic component was provided by isolation of a factor from M. hyopneumoniae membranes cytopathic for primary pig lung fibroblasts and a human lung fibroblast cell line (Geary and Walczak, 1983 and 1985). The role of this membrane component in pathogenesis of the disease is not known.

Airway mucus secretion and disease. Mucopurulent exudate in small airways is a prominent feature of M. hyopneumoniae infection in pigs (Ross, 1986). A previous study of pig

bronchial epithelium demonstrated that marked changes in bronchial goblet cells and submucosal glands occurred 3 to 5 weeks after inoculation with porcine mycoplasmas (Jones et al., 1975).

Goblet cell and submucosal gland secretions contribute a significant portion of the components in the mucous blanket. A qualitative or quantitative change in the secretions produced by these cells could cause a change in the mucociliary blanket, and therefore in the immediate environment of the cilia. Spicer et al. (1983) suggested that changes in glycoproteins of the ciliary glycocalyx could cause cilia to stick together, rather than being mutually repulsive which is theorized to be the normal state of the ciliary surface. Clumping of cilia, frequently observed histologically in bronchi of pigs infected with M. hyopneumoniae, might be caused by changes in the glycoprotein composition of the ciliary glycocalyx.

The nature of the ciliary surface is also determined by glycoconjugates present on ciliated cells in airway surface epithelium, as well as the mucociliary blanket. Synthesis of sulphated glycoconjugates, transfer of the molecules to the cell surface and their subsequent release to the extracellular environment has been demonstrated with canine tracheal epithelial cells (Nadel and Borson, 1987). Therefore, glycoconjugates present on the surfaces of

ciliated cells may contribute to the mucociliary blanket.

Airway epithelial cell secretions contain glycoproteins which may be either acidic or neutral. Mucins with acidic glycoproteins contain sulphate or sialic acid, or both, and are defined as sulphomucins or sialomucins, respectively. Sialomucins may be further divided into sialidase-sensitive and sialidase-resistant mucins. Neutral mucosubstances possess terminal sugars such as N-acetylglucosamine and N-acetylgalactosamine which lack anionic groups. In pigs, the percentage of airway goblet cells producing sulphomucin increases linearly with age (Mills *et al.*, 1986).

In histologic tissue sections, Periodic acid-Schiff/Alcian blue (PAS/AB) stains acid mucosubstances such as hyaluronic acid, sialomucins and sulphated mucosubstances blue; neutral mucosubstances with terminal sugars such as N-acetylglucosamine and N-acetylgalactosamine which lack anionic groups, are stained red (Pearse, 1980). Presence of D-galactose, L-fucose, N-acetylglucosamine or N-acetylgalactosamine in tissue components not generally classified as carbohydrate-containing may cause positive staining reactions (Pearse, 1980). High iron diamine/Alcian blue (HID/AB) stains sulfomucin (sulfate esters) black while sialomucins and uronic acid-containing mucosubstances appear blue (Pearse, 1980; Spicer *et al.*, 1983).

Quantitation of mucin within airway epithelial cells has

been assisted by the development of computerized analysis. Adler et al. (1981a) quantitated the percentage of the area of surface mucosa and submucosal glands of porcine tracheal organ cultures that stained with PAS/AB using an automated image analyzer. The total area of the mucosa in the section, as well as the mucosal area consisting of PAS/AB-staining material, was determined. This gave a quantitative measure of mucin retained in the mucosal and submucosal gland cells.

Jones et al. (1975) induced enzootic pneumonia in pigs with a mycoplasma culture identified as M. hyorhinis. Bronchial goblet cells of control pigs were well defined and chalice shaped, and contained predominantly acid glycoprotein. A single goblet cell sometimes produced both neutral and acid glycoprotein. Goblet cells of mycoplasma-infected pigs were not well defined, depleted in number and irregularly distributed within the hypertrophied bronchial epithelium. Additionally, goblet cells of infected pigs were depleted of secretion and contained predominantly neutral rather than acid glycoprotein. However, sulfomucin, an acid glycoprotein, was significantly increased in bronchial goblet- and submucosal gland-cells in infected pigs when compared to those of controls (Jones et al., 1975).

When compared to uninoculated controls, the submucosal glands of infected pigs were hypertrophied in response to mycoplasma infection (Jones et al., 1975). Hyperplasia of

bronchial epithelium in pigs has been reported as early as 7 days after inoculation with porcine mycoplasma (Baskerville, 1972). When porcine tracheal organ cultures inoculated with M. hyopneumoniae were compared to controls, thickening of the epithelium was observed (Pi Joan et al., 1972). Six weeks after inoculation with M. hyopneumoniae metaplasia of bronchiolar epithelium, accompanied by a change in lectin affinity, was reported (Ackermann et al., 1991).

Jones et al. (1975) suggested that changes in the proportion of transferases may be an important factor in the modulation of glycoprotein synthesis by porcine mycoplasma. Perhaps M. hyopneumoniae secretes a product that participates in signal transduction and alters the level or activity of selected transferases in respiratory secretory cells. Extracellular proteinases from neutrophils, mast cells and bacteria increased the rate of release of synthesized glycoconjugates to the environment (Nadel and Borson, 1987). Furthermore, some inflammatory mediators (basic polypeptides such as kinins) may function as modulators of certain glycosyltransferases, by interacting with some component of the membrane to which the enzymes are attached, thereby changing the oligosaccharide component of the mucin molecules being manufactured. Maximal activity of galactosyltransferase, a manganese-dependent enzyme, was obtained in canine tracheal explants with suboptimal

concentrations of manganese when basic polypeptides were in the incubation mixture (Baker et al., 1977).

Increased mucus secretion by cystic fibrosis patients may be induced by bacterial colonization of airways (reviewed in Reid et al., 1983). Mucin secretion by tracheal tissue was stimulated in vitro by bacterial products such as cholera toxin (Adler et al., 1981b), proteinases (Boat et al., 1984; Klinger et al., 1984) and cell free filtrates of broth cultures of Pseudomonas aeruginosa (Adler et al., 1983). Since sulphation increases with hypersecretion (Lamb and Reid, 1968), increased sulphomucin in goblet cells and submucosal glands of infected pigs (Jones et al., 1975) may reflect hypersecretion in response to infection with porcine mycoplasmas. Intimate association of M. hyopneumoniae with respiratory epithelium in vivo (Mebus and Underdahl, 1977; Underdahl et al., 1980; Tajima and Yagihashi, 1982) would permit the organism to directly affect goblet cell function/secretion. Increased mucus observed in airways of pigs with M. hyopneumoniae infection may be due to hypersecretion of mucus as well as disruption of the ciliary escalator.

Increased secretion of sulfomucin may also be induced in response to local irritation (Lamb and Reid, 1968; Reid et al., 1983). Mycoplasma hyopneumoniae produces hydrogen peroxide (DeBey and Ross, 1988) which could precipitate local

irritation of mucosal cells. Complement activation, neutrophil degranulation and other inflammatory mediators released during the host immune response to M. hyopneumoniae probably contribute to irritation of the mucosal surface.

Alterations in pH, ionic strength and concentration can produce significant changes in viscoelastic behavior of tracheal mucus. Wide variations probably occur in vivo because such alterations have been observed in tracheal secretions collected from normal dogs (Lutz et al., 1973). Increased protein within the mucous blanket may also increase viscosity because addition of albumin enhanced the viscosity of pig gastric mucus (List et al., 1978). Failure to clear mucus from the airways may be caused by an inability of the airway epithelia to secrete chloride (Widdicombe, 1987) reducing the fluid content of the mucous blanket, and thereby increasing viscosity.

Increased calcium levels in mucus have been associated with increased viscosity. Rehydrated mucin molecules collapse under the influence of calcium, that is, the presence of calcium reduces the size of the mucin polymer particles and the hydrodynamic radius of larger polymers (Steiner et al., 1984). Calcium has been found in large amounts in mucin secretory granules. In vitro studies indicated that mucins in mammalian goblet cells expand by swelling during or after exocytosis. Increased extracellular

calcium in concentrations similar to those found in the mucus of cystic fibrosis patients produced a four-fold decrease in the rate of swelling of newly released mucin polymer, resulting in mucus that remained thick for a prolonged time. In newly released mucins the polyionic charges control the rate of swelling and hence the rheology of mucus; once the mucin polymer network is fully expanded the effect of electrostatic interactions and their effect in mucus rheology is not as pronounced (Verdugo et al., 1987).

Sulphation of mucin molecules, which occurs post-translationally in the Golgi apparatus, may or may not affect rheology of the mucous blanket. Marriott and Irons (1974) found no correlation between elasticity or viscosity and sulphate content of salivary mucus, even though they demonstrated that a sulphated glycoprotein was involved in gel formation. However, Mian et al. (1982) demonstrated that N-acetylneuraminic acid content correlated with low viscosity and sulphate ester content correlated with high viscosity of mucins. An increase in the highly sulphated component of mucin has been correlated with increased viscosity of tracheobronchial mucus secretion in cystic fibrosis patients (Chace et al., 1983). However, mucus from airways of normal individuals or those infected with bacterial pathogens may contain components such as DNA which contribute to increased viscosity (Lieberman, 1968) and confound attempts to

correlate sulphated component content with rheology.

Sulphation of mucus may increase the lifetime of some species of glycoproteins (Mian *et al.*, 1979a, 1979b). The contribution of increased longevity of glycoproteins to their role as receptors for secondary bacterial invaders is unknown. Adherence to receptors in the mucus layer may enhance colonization of mucosal surfaces by microorganisms (Freter, 1988). Additionally, penetration of bacteria to the mucosal cell surface may be augmented by planes of stress in the mucus layer created during extrusion of goblet cell contents (Freter, 1988), which is presumably more frequent during hypersecretion. The effect of changes in goblet cell mucin carbohydrate on mycoplasma colonization or survival is unknown.

Changes in porcine respiratory mucin carbohydrate in response to mycoplasma infection may be a factor in accumulation of mucopurulent exudate in infected airways. Altered viscoelasticity might hinder removal of particulate matter from airway epithelium, thereby enhancing mycoplasma colonization and damage to epithelium by inflammatory cells. Dysfunction of the mucociliary escalator could also hinder removal of resident bacteria from the epithelial surface, leading to secondary bacterial pneumonia, and increased morbidity and mortality of the infected population.

A major question still to be resolved is the mechanism

by which porcine mycoplasmas might induce alterations in the character or quantity of mucin produced or released by airway goblet cells. Changes in porcine respiratory goblet cell mucin carbohydrate in response to mycoplasma infection may be indicative of elaboration of diffusible substances which affect mucin-producing cells. Perhaps porcine mycoplasmas secrete a product that participates in signal transduction and alters the level or activity of selected transferases in respiratory secretory cells.

Use of tracheal ring explants for study of mycoplasmal mechanisms of pathogenicity. Tracheal explant culture is the oldest and most widely used respiratory tract model system (Gabridge, 1986). Each explant section contains multiple cell types which are similar in relationship, structure, and function to those found in vivo.

Ciliostasis is a common manifestation of epithelial cell damage in tracheal ring cultures. The mechanisms by which ciliostasis is induced by most mycoplasmas are not understood.

Sanderson and Dirksen (1986) reported that ciliated cells of rabbit tracheal explants were sensitive to mechanical stimulation, and transmitted this response to adjacent cells. Because transmission of the response across one cell boundary required approximately 3 seconds, the authors speculated that an intracellular messenger was

transmitted by means of gap junctions. Regulation of mucociliary clearance may be influenced by mechanical interactions between cilia and mucus, because ciliated cells can increase ciliary activity in response to pressure on the ciliary membrane (Sanderson and Dirksen, 1986).

Elevated extracellular calcium caused ciliary reversal in protozoa (Naitoh and Kaneko, 1972), but did not significantly affect the ciliary activity of rabbit tracheal cilia (Girard and Kennedy, 1986). However, sensitivity of ciliated cells of rabbit tracheal explants to mechanical stimulation was lost in the absence of extracellular calcium (Sanderson and Dirksen, 1986). Increased intracellular calcium, mediated by the calcium ionophore A23187, induced an initial increase in ciliary beat frequency, leading to a time of steady slowing of ciliary beat frequency, accompanied by ciliary dyskinesia. Cells were viable for variable times after ciliary stasis occurred (Girard and Kennedy, 1986).

Tracheal organ cultures have been useful for study of damage induced to the mucosal surface by epithelial pathogens such as mycoplasmas. For studies in which ciliary integrity and activity were directly evaluated, the trachea was cut into rings for in vitro culture, and ciliated epithelium was assessed microscopically. The most common methods for evaluation of ciliated epithelium involve measurement of ciliary activity or evaluation of the integrity of tracheal

epithelium (Gabridge, 1986).

Measurement of ciliary activity is commonly accomplished by visually assessing ciliary movement using an inverted microscope, and assigning a numerical score for vigor of ciliary beating. Assignment of a score to describe vigor of ciliary beating for each ring (Gabridge and Polisky, 1976; Chandler and Barile, 1980) was the usual procedure adopted for measurement of ciliostasis induced by mycoplasmas. The disadvantage of assigning one ciliary activity score per ring is that all areas of tracheal ring epithelium may not exhibit the same vigor of ciliary beating after inoculation with mycoplasma. However, assigning a single score for ciliary activity produced acceptable estimates of the vigor of ciliary beating (Gabridge and Polisky, 1976; Chandler and Barile, 1980).

Use of a computerized electro-optical system to measure ciliary activity is more sensitive than subjective visual evaluation. When ciliary motion was monitored by light microscopy no reduction in ciliary activity was observed after infection by Chlamydia trachomatis (Luzzatto et al., 1989). However, measurement with a computerized electro-optical system in the same study revealed that about 50% of the cilia of infected cells were paralyzed at 48 hours post infection, and cilia not paralyzed showed a 40% decrease in beating frequency at that time (Luzzatto et al., 1989).

Discrepancies between visual and computer aided evaluation are not surprising. Visual examination of both infected and non-infected cells can mask the ciliostatic effect of pathogens because non-infected, normally beating cells contribute to the final score. Computerization allows measurement of an individual cell, but the measuring apparatus must be accurate and functioning properly, and much data is needed to obtain a reliable average (Luzzatto et al., 1989).

Subjective estimation of the integrity of tracheal ring epithelium by observing the percent of epithelium retaining cilia may be accomplished using an inverted microscope. To objectively evaluate integrity of tracheal epithelium, assessment of the quantity or function of normal cell constituents may be measured.

Infection of hamster tracheal organ cultures with virulent M. pneumoniae apparently leads to decreased cellular activity within the explant. Several cell components have been measured to assess the effect of M. pneumoniae on hamster tracheal ring epithelium. Inoculation of hamster tracheal rings with M. pneumoniae caused significant loss of ciliary activity and decreased dehydrogenase enzyme activity (Gabridge and Polisky, 1976), ATP content (Gabridge and Polisky, 1977), and oxygen utilization (Gabridge, 1975) by the explants when compared to controls. These results were

compatible with those reported by Hu and Baseman (1975).

Infection of tracheal organ cultures by virulent M.

pneumoniae caused a decrease in uptake of amino acids, orotic acid, and galactose as well as a decrease in protein and ribonucleic acid (RNA) synthesis.

Another cell component used for assessing explant epithelial cell damage is calmodulin. Calmodulin, a calcium binding protein found in most eukaryotic cells, is the major receptor for calcium and modulates most of the ion's activities. Within cells calmodulin is found free in the cytoplasm or associated with the cell membrane or organelles (reviewed in Klee et al., 1980). The amino acid sequence of this heat- and acid-stable protein is highly conserved. Calmodulin has been shown to regulate cellular motility, cyclic nucleotide levels, metabolic pathways and intracellular calcium fluxes. Calmodulin activates the calcium pumps of the plasma membrane and the sarcoplasmic reticulum, both of which are responsible for controlling cellular concentrations of calcium (reviewed in Klee et al., 1980). Verdugo et al. (1983) reported that calmodulin played a role in the regulation of ciliary movement of mammalian ciliated cells. An inhibitor of calmodulin, trifluoroperazine, arrested ciliary movement of ciliated cells in rabbit tracheal organ cultures.

Bermudez et al. (1990) measured calmodulin content in

equine oviduct explants to evaluate the integrity of explant epithelium after inoculation with Mycoplasma equigenitalium. Calmodulin content in equine oviduct rings inoculated with Mycoplasma equigenitalium was lower than values of corresponding controls. Bermudez et al. (1990) observed that ciliostasis and decreased intracellular calmodulin level were highly correlated.

Porcine tracheal rings have been employed for pathogenicity studies of porcine pathogens including mycoplasmas (Reed, 1971; Pijoan et al., 1972; Williams and Gallagher, 1978; Rosengarten and Kirchhoff, 1981; Williams et al., 1981; Belanger et al., 1990). Additionally, mucin composition and secretion have been evaluated using porcine trachea or tracheal rings (Adler et al., 1981a; Turek et al., 1982; Chandrasekaran et al., 1984; DeBuysscher et al., 1984; Gabridge, 1984; Lloyd et al., 1984; Rana et al., 1987).

Progressive ciliostasis and epithelial exfoliation in porcine tracheal rings co-cultivated with fetal porcine lung fibroblasts and M. hyopneumoniae was reported by Williams and Gallagher (1978). However, most workers observed that M. hyopneumoniae caused no observable ciliostasis or loss of cilia in vitro (Pijoan et al., 1972; Cherry and Taylor-Robinson, 1973; Rosengarten and Kirchhoff, 1981). Pijoan et al. (1972) reported that M. hyopneumoniae enhanced the longevity of ciliary activity of tracheal organ cultures.

The ability to colonize epithelial cells of tracheal rings and the nature of cytotoxic effects of mycoplasmas in vitro depends on 3 factors: culture conditions, host tissue, and status of the mycoplasma culture. Proper control of these factors is necessary to develop an in vitro model which accurately reflects pathogenic mechanisms operative in vivo.

Alterations in culture conditions may be attained by varying the concentration of CO₂ in the environment, changing media formulations, varying serum type and content, and replenishing spent medium at various times. The advantages and disadvantages of providing medium permissive for pathogen growth should be considered when developing an in vitro model for study of pathogenesis of mycoplasmal infection. When using explant medium permissive for mycoplasma growth, one risks inducing indirect, possibly artifactual effects resulting from accumulation of toxic metabolites, pH alterations, and/or nutrient or oxygen depletion (Gabridge and Stahl, 1978b). Gabridge and Stahl (1978b) reported that M. pneumoniae infection of hamster tracheal explants produced significantly greater ciliostasis and cytonecrosis in a "permissive" medium capable of supporting mycoplasma metabolism and replication, than in a "nonpermissive" medium. With a permissive medium cell damage may result from mycoplasmas which are free living in the medium as well as mycoplasmas which are attached to the host cell membrane.

Gabridge and Stahl (1978a) demonstrated that radiolabeled adenine in host cells was incorporated into adherent M. pneumoniae. They suggested that M. pneumoniae scavenged adenine and induced cell damage by reducing the adenine available for nucleotide synthesis in the host cell. When tracheal explants were maintained in medium permissive for M. pneumoniae growth, no protection against cell damage was detected with adenine supplementation. However, when a nonpermissive medium was used as the post-infection explant medium adenine provided striking protection (Gabridge and Stahl, 1978a, 1978b). Hu et al. (1975) demonstrated significant differences in RNA and protein synthesis only when infected explants were incubated in a medium permissive for mycoplasma growth. These results may indicate additive or synergistic effects of free-living and attached organisms (Gabridge and Stahl, 1978b), or may indicate that the most toxic organisms are those which are able to rapidly proliferate. Cell damage noted under permissive conditions in vitro may result from processes distinct from those operative during host-parasite interaction in vivo (Gabridge and Stahl, 1978b).

With a nonpermissive medium the observable cell damage results from the intimate association of extracellular, membrane-bound mycoplasmas on the surface of host epithelial cells (Gabridge and Stahl, 1978a). However, nonpermissive

medium may not be adequate to maintain optimum viability and metabolic activity of host cells and/or fastidious pathogens. Almagor et al. (1983, 1984) reported that viable M. pneumoniae organisms were necessary to generate the superoxide anion that inhibited host cell catalase.

A second factor to consider when developing an in vitro model of pathogenicity is the nature of the host cells, including species and tissue of origin. Additionally, use of explants vs. cell monolayers, primary cell cultures vs. cell lines, and physical or chemical treatment of host tissue before inoculation with mycoplasmas can alter cell structure and function. Cultivation in vitro in various media or for various times before inoculation may alter host cell susceptibility to the pathogen of interest. Upchurch and Gabridge (1981) reported that the outcome of M. pneumoniae infection in vitro depended upon the metabolic state of host cells. Cells maintained in 20% serum were more metabolically active and more likely to exhibit changes in metabolic status in response to M. pneumoniae infection than cells maintained in medium with a lower serum content.

Metabolic status or cytotoxic potential of the mycoplasma culture can be altered by using various strains, passage levels, and incubation times. Young 24-48 hour cultures of M. pneumoniae caused greater damage to ciliated epithelium than did 72-96 hour cultures, indicating that

metabolic/nutritional status of M. pneumoniae is important during interaction with tracheal explant cultures (Gabridge and Stahl, 1978b).

Comparisons between in vivo vs. in vitro passaged organisms may also be made. In vitro growth for as few as 6 passages led to loss of pathogenicity by M. hyopneumoniae for pigs (Tajima and Yagihashi, 1982). Therefore, failure of M. hyopneumoniae to elicit ciliostasis in porcine tracheal rings might be due to loss of virulence during passage of the organism in vitro. Loss of virulence in vitro might be due to loss of stimuli recognized by M. hyopneumoniae in vivo when associated with the respiratory mucosa of a pig.

The role of hydrogen peroxide or hemolysin in the pathogenesis of mycoplasmal disease is unresolved because of conflicting reports on the subject (Cole et al., 1968; Yayoshi, 1983; Almagor et al., 1984; Minion and Goguen, 1985; Yayoshi et al., 1985; Davidson et al., 1988). Mycoplasma pneumoniae, which causes pneumonia in humans, hemolyzed erythrocytes by the action of hydrogen peroxide (Cole et al., 1968; Sobeslavsky and Chanock, 1968; Cohen and Somerson, 1969a, 1969b). Hydrogen peroxide produced by mycoplasmas has been incriminated as a factor cytotoxic for tracheal explant epithelium. Cherry and Taylor-Robinson (1970) demonstrated that tracheal explants infected with M. mycoides subsp. capri exhibited greater cytotoxicity and hydrogen

peroxide content than uninoculated controls. Catalase protected ciliated epithelium from damage. Additionally, Simon et al. (1981) demonstrated that hydrogen peroxide was the damaging oxygen species when lung fibroblasts were exposed to superoxide, hydrogen peroxide, hydroxyl radical or singlet oxygen. Damage to equine oviductal epithelial cell membranes by hydrogen peroxide produced by M. equigenitalium was suggested as a possible mechanism for release of host intracellular calmodulin to the extracellular environment in this model (Bermudez et al., 1990).

Conversely, Lipman and Clyde (1969) reported that the quantity of peroxide produced by two strains of M. pneumoniae was not related to virulence. Yayoshi (1983) reported that a non-hemolytic, chemically mutagenized strain produced significantly milder lung lesions than the hemolytic parent strain. Possibly more than one gene was interrupted by the chemical mutagenesis. Davidson et al. (1988) reported that there was no relationship between hemolytic activity and pathogenicity of M. pulmonis. Hydrogen peroxide production by mycoplasmas may not be related to virulence, but may rather reflect the ability of the strain to grow and metabolize in vitro since it is a common by-product of synthetic pathways (Chance et al., 1979).

Mycoplasma hyopneumoniae caused hemolysis of turkey red blood cells upon prolonged incubation in hemagglutination

assays (Young and Ross, 1990). Possibly, this hemolysis was caused by hydrogen peroxide or another hemolysin produced by the mycoplasma. Because the association between M. hyopneumoniae and respiratory epithelium is intimate, production of small amounts of hydrogen peroxide by the organism may result in host cell injury. Pijoan (1974) reported that 2 strains of M. hyopneumoniae failed to produce hydrogen peroxide, because human erythrocytes exposed to the organism failed to stain with methylene blue. Staining of erythrocytes with methylene blue is a direct method for detection of hydrogen peroxide (Lind, 1970). However, some direct methods may not be as sensitive as indirect methods, such as those using catalase and triazole, for detection of very low levels of hydrogen peroxide (Cherry and Taylor-Robinson, 1970).

An assay utilizing catalase and triazole can be used to detect production of low levels of hydrogen peroxide over a relatively long period of time (Margoliash et al., 1960). Catalase, an enzyme present in animal cells, blocks peroxide damage to cell membranes by binding to hydrogen peroxide to form Complex I (Chance et al., 1979). The ultimate outcome of further reactions is the generation of water and oxygen, and release of free catalase enzyme to the environment (Cohen and Somerson, 1969a). The enzymic activity of catalase may be irreversibly inhibited by binding of 3-amino-1,2,4-

triazole (triazole) to Complex I (Margoliash et al., 1960). Therefore, inhibition of catalase by triazole is dependent upon hydrogen peroxide binding to the catalase (Margoliash et al., 1960; Cohen and Somerson, 1969a).

Mycoplasma pneumoniae inhibits host cell catalase (Almagor et al., 1983) which allows hydrogen peroxide generated by the mycoplasma and host cell to increase intracellularly and cause oxidative damage to the cell. Superoxide anion has been implicated in host cell injury inflicted by M. pneumoniae as the inhibitor of host cell catalase (Almagor et al., 1984), probably by conversion of the iron ion of heme in catalase to the inactive state (Kono and Fridovich, 1982). Ability of M. hyopneumoniae to produce superoxide anion has not been determined.

Levels of malonyldialdehyde (MDA) indicate the amount of lipid peroxidation in cultured cells. Addition of catalase to cells infected with M. pneumoniae did not affect MDA levels (Almagor et al., 1984). However, MDA levels were reduced in cell lines to which superoxide dismutase was added. Reduction of MDA levels in cell lines supplemented with superoxide dismutase was cited as evidence that superoxide anion damaged cells infected with M. pneumoniae (Almagor et al., 1984).

Depletion of nutrients by mycoplasmas scavenging for molecules necessary for growth or metabolism, or by enzymes

secreted by mycoplasmas has been demonstrated in vitro. Arginine depletion is associated with pathogenicity in cell cultures infected or contaminated with mycoplasmas which produce arginine deiminase (reviewed in Gabridge et al., 1985). Nucleoside triphosphate content (Upchurch and Gabridge, 1982) and de novo purine synthesis were decreased in fibroblasts infected with M. pneumoniae (Upchurch and Gabridge, 1983).

When hamster tracheal explants were incubated with radiolabeled adenine, then inoculated with M. pneumoniae, the adenine label was recovered from the mycoplasmas 20 hours after infection (Gabridge and Stahl, 1978a). These data support a model for depletion of host cell nucleic acid precursors as a mechanism for cytotoxicity in tracheal explants. Depletion of nucleosides or nucleic acid precursors could impair or kill affected host cells.

Depletion of molecules necessary for host cell function as a mechanism of cytotoxicity is apparently common among mycoplasmas. However, toxins have been partially characterized from several mycoplasma species. The toxins produced by some mycoplasmas are formed only when the mycoplasmal cell membrane is intact and/or the organism is in an active stage of metabolism.

One of these toxins is produced by M. neurolyticum, which causes "rolling disease" in mice. The cell free,

proteinaceous exotoxin is somewhat species specific, causing neurological signs in young mice and rats, but not in non-murine rodents. The apparent molecular weight (M.W.) is greater than 200,000, but lability of the toxin has hampered characterization or elucidation of the mechanism of action (reviewed in Gabridge et al., 1985). Neurotoxic activity of M. gallisepticum (strain S6) for young turkey poults has been demonstrated with broth cultures. No evidence of cell free exotoxin in culture supernatant was detected, but intact viable cells were necessary for production of toxic activity (Thomas and Bitensky, 1966; reviewed in Gabridge et al., 1985).

Diffusion chambers containing M. mycoides subsp. mycoides elicited significantly greater host fibrinous and cellular response than control chambers in rabbits and cattle (Lloyd, 1966; Buttery et al., 1980). In these models, diffusible toxin or substances produced by M. mycoides subsp. mycoides induced encapsulation which may be a mechanism to protect the organisms from cellular host defenses during infection (Gabridge et al., 1985). Galactan of M. mycoides subsp. mycoides was prepared from the extracellular polysaccharide which was present in culture supernate after removal of mycoplasma cells by centrifugation (Buttery et al., 1976). A galactan obtained by phenol extraction of M. mycoides cells was composed of approximately 90% galactose,

4% lipid, 2% nitrogen and 0.1% phosphorus (Plackett and Buttery, 1958; Villemot et al., 1962; reviewed in Gabridge et al., 1985). Buttery et al. (1976) suggested that respiratory distress and pulmonary lesions exhibited by calves inoculated with the preparation were possibly due to release of vasoactive amines by vascular cells in response to the galactan.

Lipoglycans isolated from acholeplasma are structurally dissimilar to, but mimic the action of, endotoxin (Seid et al., 1980). The presence of lipoglycans in mycoplasmas is not always associated with virulence (reviewed in Gabridge et al., 1985). These substances may be ancillary factors in pathogenesis, rather than primary means of pathogenicity.

A complex polysaccharide (M.W. 73,000) produced by M. bovis is apparently contained within a complex glycoprotein in the mycoplasmal cell. The polysaccharide toxin caused clinical mastitis when injected into bovine mammary glands. Ability of the toxin to activate complement, thereby altering vascular permeability, may enhance dissemination of the organism in mammary tissue (Geary et al., 1981).

Presence of enzymes that might be related to pathogenicity of various mycoplasmas has been investigated. Degradation of collagen would seem to be a likely mechanism for induction of arthritis by M. arthritidis in rats (reviewed in Gabridge et al., 1985). Greatly increased

collagenase activity of cultured murine cells (BALB/c 3T3 fibroblasts) was observed when those cells were contaminated with M. orale (Kluve et al., 1981), indicating that induction of collagenolytic activity by cells upon infection with mycoplasmas may be important in development and persistence of connective tissue disease (Gabridge et al., 1985).

Strains of Ureaplasma urealyticum associated with spontaneous abortion and perinatal morbidity and mortality exhibited high levels of phospholipase activity (De Silva and Quinn, 1986 and 1987). The authors suggested that microbial phospholipases may participate in the initiation of premature labor. Increased phospholipase activity may induce premature labor by hydrolysis of placental membrane phospholipids, leading to increased free arachidonic acid and synthesis of prostaglandins. Additionally, lysophospholipids released by phospholipases may exert a hydrolytic effect on membranes (De Silva and Quinn, 1986 and 1987). Phospholipases have not been isolated and identified in M. hyopneumoniae. However, lipase activity was detected in M. hyopneumoniae, but not in M. flocculare, a closely related porcine respiratory commensal (Kies et al., 1991). The importance of lipase activity for pathogenicity is not known.

When IgA protease activity has been detected in mycoplasma species, cleavage of IgA from the natural host only is apparent. Possibly, this factor may contribute to

host specificity of mycoplasmal pathogens (reviewed in Gabridge et al., 1985).

Aminopeptidases have been demonstrated in Ureaplasma, Mycoplasma and Acholeplasma species. Aminopeptidase activity did not correlate with pathogenicity of porcine (Kies et al., 1991) or bovine mycoplasmas (Neill and Ball, 1980). Liquefaction of gelatin and coagulated serum has been described for several species. However, in spite of documentation that mycoplasmas produce several enzymes which have potential to cause tissue damage, their role in disease pathogenesis is not known (reviewed in Gabridge et al., 1985).

Toxic components possessed by mycoplasmas are often located in the membrane of the organism. Membrane preparations of M. fermentans were toxic for mice. Lipid and protein moieties of the membranes were required for toxicity (Gabridge and Murphy, 1971; Gabridge et al., 1972). Ciliostasis was induced by M. pneumoniae membranes (Gabridge et al., 1974). Method of membrane preparation, conditions under which cells are exposed to toxin, and age of mycoplasma culture are all potential variables contributing to the lack of agreement between reports on toxicity of mycoplasmal membranes (Gabridge et al., 1985).

Induction of pneumonic lesions by nonviable mycoplasma membranes has been demonstrated in mice (reviewed in Gabridge

et al., 1985) and pigs (Messier and Ross, 1986). Correlation between mitogenicity and pathogenicity of M. pulmonis membranes suggested that the mitogenicity of M. pulmonis may play a role in murine mycoplasmal pneumonia. However, Davidson et al. (1988) were unable to find a relationship between mitogenicity and pathogenicity of 18 strains of M. pulmonis. Mitogenic activity has been described for several mycoplasma species. Mitogenic entities may be associated with membrane structural components or be released into extracellular medium. Variations in the composition of mitogenic factors as well as their specificity for T or B cells have been described (reviewed in Gabridge et al., 1985).

Toxicity of broth passaged M. hyopneumoniae for cell culture systems has been demonstrated. Whole organisms of M. hyopneumoniae induced no cytopathic effect in rabbit kidney primary tissue cultures in an early study (Pijoan, 1975). Chen et al. (1981) later described a dose relationship between membrane preparations of M. hyopneumoniae and cytotoxicity for rabbit kidney primary cells (Chen et al., 1981). Cultures were inoculated with 6-50 ug membrane protein. Cells receiving 6-17.5 ug membrane protein exhibited no significant cytopathic effect. However, cells inoculated with 25-50 ug membrane protein exhibited significant cytopathic effects when compared to control (uninoculated) cells.

Whole M. hyopneumoniae cells were cytotoxic for primary cultures of porcine lung fibroblasts derived from 1 day old piglets and for the MRC-5 human lung fibroblast cell line (Geary and Walczak, 1983). Cytotoxicity of nonviable M. hyopneumoniae membranes indicated that damage to cell monolayers was not due to depletion of essential nutrients or the build-up of toxic metabolic end products by viable M. hyopneumoniae. In contrast to the report of Chen et al. (1981), 2 ug of membrane protein, or 250 ng/ml of cytopathic factor isolated from the mycoplasma membranes, were sufficient to induce cell damage in MRC-5 cells. The cytopathic factor appeared to be antigenic, since rabbit anti-M. hyopneumoniae serum was capable of neutralizing its cytopathic activity. This factor was not attenuated or lost after numerous passages in broth. The amount of cytopathic factor in high and low passage M. hyopneumoniae J appeared to be equivalent. The factor was heat labile (100 C for 15 minutes) and destroyed by pronase. Cytoplasm of M. hyopneumoniae was not cytotoxic for these cell systems (Geary and Walczak, 1985).

Because of the intimate association of mycoplasmas with host cells, some workers have suggested that fusion between the mycoplasmal and eukaryotic membranes may occur. However, morphologic evidence of fusion between mycoplasmal and host cell membranes is inconclusive because artifactual changes

during fixation and processing are difficult to rule out. Fusion with lipid vesicles and transfer of antigens between mycoplasmas and cells in culture are biological evidence of membrane fusion (reviewed in Gabridge et al., 1985).

As a mucosal pathogen, *M. hyopneumoniae* is closely associated with the plasma membranes of colonized cells. The attachment of *M. hyopneumoniae* to host cell membranes may alter normal membrane function. If *M. hyopneumoniae* utilizes cell components, such as phospholipids and fatty acids from the host cell membrane, significant alterations in membrane structure and function could result. Activation or blockage of key intrinsic membrane proteins by attached mycoplasmas could cause alterations of intracellular concentration or regulation of key messenger molecules, such as cAMP and calcium. Identification of the host cell receptor molecule may facilitate elucidation of pathogenic mechanisms of this organism.

The mycoplasmas apparently utilize a number of mechanisms to induce mucosal cell damage. Study of mechanisms of pathogenicity have been advanced by elucidating interactions of mycoplasmas with cell and organ cultures in vitro. Potent cytotoxins are apparently produced by some, but not all, mycoplasmal species. However, subtle mechanisms for cytotoxicity are commonly observed, and may be sequelae of mycoplasmal mechanisms for survival on the host mucosa.

Cytoplasmic calcium flux and neutrophil activation as mechanisms of cell injury. Early microscopic changes in swine mycoplasmal pneumonia include accumulation of neutrophils in and around airways and in alveoli. Large numbers of neutrophils are commonly observed in bronchiolar lumina in early and mid-stage disease (Livingston et al., 1972; Ross, 1986), coinciding with apparent immunosuppression during that time (Adegboye, 1978a, 1978b; Ross, 1986). During lesion resolution neutrophils disappear from airways (Ross, 1986). The percent of peripheral blood neutrophils was increased during late mid-stage to early advanced stage of infection, 4-6 weeks after infection (Intraraksa et al., 1984). The neutrophilic content of pulmonary lesions in swine is often influenced by secondary invasion with other bacteria (Gois et al., 1975; Ross, 1986).

The role of neutrophils in pathogenesis of M. hyopneumoniae disease is not known. Neutrophils, recruited during the time of greatest lesion severity, may assist host clearance mechanisms or may contribute to host cell injury. Accumulation of neutrophils in airways (Livingston et al., 1972) coincides with epithelial cell injury during acute and mid-stage disease. In humans, the secretory products of neutrophils have been implicated in cell injury observed during respiratory distress syndrome (Till et al., 1982). However, the role of neutrophils in mediating lesion

development or resolution during mycoplasmal pneumonia is not known.

Leukocytes adhering to bronchial epithelium after inoculation of pigs with broth cultures of M. hyopneumoniae appeared to have mycoplasmas adhering to their surface (Mebus and Underdahl, 1977; Underdahl et al., 1980). Adhering mycoplasmas could modulate leukocyte response to signal transduction at the cell membrane, possibly leading to inappropriate activation of leukocytes, including neutrophils. Activated neutrophils typically produce oxygen metabolites (hydrogen peroxide, superoxide anion, hydroxyl radical) which are capable of killing type II pneumocytes, leading to reduced surfactant production (Crim and Simon, 1988). Cellular function of murine type II pneumocytes was impaired by concentrations of oxygen metabolites equivalent to the amount produced by in vivo neutrophil:type II pneumocyte ratios of 3:1 to 6:1 (Rasmussen et al., 1985). Previously reported evidence of reduced function of the surfactant system in the lungs of pigs infected with M. hyopneumoniae (Wichert and Wilke, 1976) may indicate impaired cellular function of type II pneumocytes which were damaged by oxygen metabolites produced by adjacent neutrophils. Impaired cellular function may result in reduced production of surfactant by type II pneumocytes (Crim and Simon, 1988). However, edema observed in mycoplasma affected lungs could

alter function of the surfactant system concomitant with normal surfactant production (Wichert and Wilke, 1976).

Activated neutrophils usually exhibit higher concentrations of intracellular calcium ($[Ca]_i$) than control cells (Rasmussen et al., 1985). Detection of $[Ca]_i$ is possible with fluorescent indicators. Fura-2/AM (pentaacetoxymethyl ester; Fura-2) is a fluorescent indicator developed for measurement of cytosolic free calcium concentration in cells (Grynkiewicz et al., 1985), and was used for measurement of intracellular calcium flux in bovine neutrophils in response to various neutrophil stimulants (Brown and Roth, 1991). During incubation of intact cells with the membrane-permeant ester derivative of Fura-2, the fluorescent indicator enters the cytosol through the cell membrane. Cytosolic esterases remove ester groups, leaving membrane-impermeant Fura-2 inside the cell (Grynkiewicz et al., 1985). Peak absorbance of Fura-2 shifts from 380 nm to 340 nm after binding to calcium, and emits light at 510 nm upon excitation at either wavelength. Measurement of light emitted at 510 nm during alternate excitation at 340 and 380 nm gives fluorescence ratios from which $[Ca]_i$ can be determined (Grynkiewicz et al., 1985).

Zymosan opsonized with porcine serum stimulates porcine neutrophils (Goff et al., 1991), probably via the C3b receptor. This apparently activates a G protein to make it

accept energy from GTP. Activated G protein activates a phosphodiesterase which cleaves phosphatidylinositol-bisphosphate, usually situated in the inner leaflet of the membrane bilayer, into inositol triphosphate and diacylglycerol. Inositol triphosphate causes release of neutrophil stores of intracellular calcium from the endoplasmic reticulum. In addition to modulation of efflux of intracellular calcium from endoplasmic reticulum, activated G protein can modulate influx of extracellular calcium through calcium channels (reviewed in Sternweis and Pang, 1990) to the cytosol. Therefore, rapidly increasing $[Ca]_i$ may be due to increased flux of calcium across the cell membrane from extracellular sources, or release of calcium from intracellular stores (Rink, 1987). Increases in $[Ca]_i$ usually indicate activation of neutrophils (Rasmussen *et al.*, 1985).

Elevation of intracellular calcium concentration can induce marked changes in cellular function. Enteropathogenic Escherichia coli caused an elevation of $[Ca]_i$ in cultured HEP-2 cells (Baldwin *et al.*, 1991). The author speculated that increased $[Ca]_i$ in enterocytes would result in the characteristic attaching and effacing lesion seen in E. coli disease by calcium-dependent activation of actin-depolymerizing proteins. Gelsolin is an actin-depolymerizing protein representative of actin modulating proteins present

in most eukaryotic cells. Elevated $[Ca]_i$ causes gelsolin to sever actin filaments and associate with filament ends, which prevents further actin filament growth (Yin and Stossel, 1979; Yin, 1988). In addition gelsolin can accelerate the assembly of actin monomers into filaments, a process called nucleation. Therefore, gelsolin, a calcium-regulated protein, modulates cell structure and motility (Kwiatkowski and Yin, 1987).

The activity of one or more calcium-dependent response elements may be altered without changing $[Ca]_i$ (Rasmussen *et al.*, 1985). Priming of neutrophils by diacylglycerol (Liang *et al.*, 1990) and nucleotides (Walker *et al.*, 1991) for enhanced $[Ca]_i$ flux in response to stimulants revealed that G proteins involved in the signal transduction process for increasing $[Ca]_i$ are different for various compounds. Diacylglycerol and nucleotides are plentiful in the environment surrounding dead or dying cells. Priming of neutrophils by these molecules to enhance cytoplasmic calcium flux without altering the resting $[Ca]_i$ may increase damage to tissue by the products of activated neutrophils. Priming of neutrophils could lead to over-production of oxygen radicals by neutrophils in response to inflammatory mediators (Walker *et al.*, 1991).

Three calcium-dependent response elements possibly affected by *M. hyopneumoniae* are calmodulin, G proteins and

calcium channels. Calmodulin complexed to calcium activates a number of enzymes, and may indirectly influence many cell functions. Calmodulin-dependent cellular processes include regulation of ciliary activity (Verdugo et al., 1983), cell motility (Klee et al., 1980) and depolymerization of cytoskeletal elements (Goligorsky et al., 1986); regulation of cyclic nucleotide levels (Wolff and Brostrom, 1979; Wang and Waisman, 1979; Evian et al., 1979); and modulation of $[Ca]_i$ by regulation of an ATPase responsible for extrusion of calcium to the extracellular space (reviewed in Klee et al., 1980).

G proteins can modulate calcium channels. Stimulation of calcium channels by G proteins apparently entails 2 pathways, one that utilizes cytoplasmic second messenger systems, and one limited to the membrane which may involve direct interaction between the G protein and the channel complex (reviewed in Sternweis and Pang, 1990).

Unsaturated fatty acids, including arachidonate, directly activate human neutrophil protein kinase C (McPhail et al., 1984). Activation of protein kinase C was shown to modulate ionic currents carried by calcium (DeRiemer et al., 1985; Di Virgillo et al., 1986; Farley et al., 1986; Rane and Dunlap, 1986; Wertz and MacDonald, 1987; Doerner et al., 1988; Marchetti and Brown, 1988; Linden and Routtenberg, 1989). Release of arachidonate in response to neutrophil

stimuli such as opsonized particles or calcium ionophore (A23187) may lead to activation of protein kinase C. After stimulus-induced phosphatidylinositol turnover, activation of protein kinase C may be induced by an increase in $[Ca]_i$ or release of diacylglycerol (McPhail et al., 1984), the second messenger of activation of protein kinase C (Kikkawa et al., 1982; Sekiguchi et al., 1987).

Additionally, fatty acids can modulate calcium channel function. Calcium channels are inhibited by fatty acids (McPhail et al., 1984; Sekiguchi et al., 1987; Hwang et al., 1990; Ordway et al., 1991). Modulation of calcium channel function may occur through activation of, or by direct interaction with, a channel regulatory protein. The most likely mechanism for fatty acid regulation of ion channels is interaction of fatty acids with ion channel proteins (Ordway et al., 1991). Fatty acids maximally inhibitory of calcium channel activity, myristic, oleic and linoleic acid (Hwang et al., 1990; reviewed in Ordway et al., 1991) compose approximately 47% of the total membrane lipid of *M. hyopneumoniae* (Hwang et al., 1986). The fatty acids in mycoplasma membranes are not free fatty acids, but are constituents of membrane lipids. Mechanisms by which they might become available for modulation of calcium channels in another membrane are not clear. See Table 1 for fatty acid composition of *M. hyopneumoniae* membrane lipid.

Table 1*. Fatty acid composition of total membrane lipid
from M. hyopneumoniae

Lipid	Chain length: No. of double bonds	% of total
Lauric	12:0	8.36
Myristic	14:0	7.21
Palmitic	16:0	31.13
Palmitoleic	16:1	1.61
Stearic	18:0	6.67
Oleic	18:1	32.66
Linoleic	18:2	7.08

* Modified from Hwang et al. (1986)

Incorporation of medium constituents into mycoplasma cell membranes may complicate study of the cell membrane composition of mycoplasmas (Nicolet et al., 1980). Serum, which composes 20-25% of Friis mycoplasma medium, contains free fatty acids. The concentration of free fatty acids can be as high as 0.6 mM in human serum or 0.75 mM in calf serum, and oleic acid constitutes almost half that amount (Wallach and Pastan, 1976). The effect of fatty acids present in Friis mycoplasma medium on M. hyopneumoniae membrane composition is not known.

Addition or removal of fatty acids alters membrane characteristics. Reconstitution of integral membrane proteins in defined lipids has established that mobility,

conformation and therefore function, of intrinsic membrane proteins may be influenced by the phospholipid environment around the hydrophobic region of the protein that is inserted into the lipid bilayer (reviewed in Katz and Messineo, 1981). Major proteins exposed at the surface of M. hyopneumoniae are covalently modified by lipid (Wise and Kim, 1987). The potential role of lipids or fatty acids in the M. hyopneumoniae membrane in pathogenesis of disease and host cell injury should be considered.

Fluctuation of $[Ca]_i$ within a eukaryotic cell might be induced by a number of mechanisms after M. hyopneumoniae attaches to that cell. Some calcium dependent response elements among eukaryotic cells are highly homologous. If a component or product of M. hyopneumoniae modulates a calcium dependent response element, the organism might alter the function of multiple cell types. Microscopic lesions indicate that M. hyopneumoniae alters the function of epithelial cells, lymphocytes, and possibly neutrophils and macrophages. Mycoplasma hyopneumoniae might possess a component that either directly affects multiple cell types, or activates a cascade of intercellular signaling leading to dysfunction of a variety of cells.

SECTION I: HISTOCHEMICAL AND MORPHOLOGICAL CHANGES OF
PORCINE AIRWAY EPITHELIAL CELLS IN RESPONSE
TO INFECTION WITH MYCOPLASMA HYOPNEUMONIAE

Histochemical and Morphological Changes of
Porcine Airway Epithelial Cells in Response
to Infection with Mycoplasma hyopneumoniae

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SUMMARY

Mycoplasma hyopneumoniae causes pneumonia in pigs. The effect of infection by this organism on histochemical characteristics of airway mucin within epithelial cells was studied. Seven to 10 week old pigs were inoculated intratracheally with either M. hyopneumoniae or culture broth, and lung tissues were collected from infected and control pigs 2, 4 and 6 weeks after inoculation. Tissue sections were stained with Periodic acid Schiff/Alcian blue, pH 2.5 (PAS/AB) or high iron diamine/Alcian blue (HID/AB). Histological features of randomly selected bronchi, bronchioles and submucosal glands were compared in sections stained with PAS/AB. Bronchial goblet cell sulphomucin and sialomucin were quantitated by image analysis of HID/AB sections. Bronchi and bronchioles of infected pigs contained proportionately fewer goblet cells with mucin at all stages of infection than age matched control pigs. Goblet cells in bronchi of infected pigs contained significantly less total mucin and sialomucin, and significantly more sulphomucin than goblet cells of control pigs. Increased sulphated mucin in bronchial goblet cells may reflect altered glycoprotein production or secretion in response to infection with M. hyopneumoniae.

INTRODUCTION

Mucopurulent exudate in small airways is a prominent feature of Mycoplasma hyopneumoniae pneumonia in pigs. Clumping and loss of cilia from the tracheobronchial epithelia occur in response to infection by M. hyopneumoniae, reflecting the intense colonization that occurs on this mucosal surface (1,2). Mycoplasmal pneumonia usually presents as a chronic disease in pigs, with maximal gross and microscopic lesion development 22-38 days after experimental infection (1).

In histologic tissue sections, Periodic acid Schiff/Alcian blue (PAS/AB) at pH 2.5 stains acid mucosubstances such as hyaluronic acid, sialomucins and sulphated mucosubstances blue; neutral mucosubstances with terminal sugars which lack anionic groups such as N-acetylglucosamine, N-acetylgalactosamine, galactose or fucose are stained red (3). High iron diamine/Alcian blue (HID/AB) stains sulfomucin (sulfate esters) black, while sialomucins and uronic acid-containing mucosubstances appear blue (3,4).

Goblet cell and submucosal gland secretions are major components of the mucous blanket. The objective of this study was to determine if infection with M. hyopneumoniae caused quantitative or qualitative changes in goblet cell mucin of pigs. Concurrent morphological changes in airway epithelia were also assessed.

MATERIALS AND METHODS

Pigs. Yorkshire and Hampshire pigs 7 to 10 weeks old were obtained from a barrier maintained, closed herd at the animal resource station at Iowa State University. The herd was originally established from Caesarean born, isolation reared swine. For this study pigs were housed in separate isolation rooms in groups of 6 or 7 animals, and fed a 16% protein swine grower ration without antibacterials. Pigs were randomly distributed into two groups on the basis of litter and size. Sera collected from each pig before inoculation and weekly after inoculation until necropsy were evaluated for the presence of antibody to M. hyopneumoniae by the complement fixation test (CF) (5).

Inoculum. Pigs were inoculated intratracheally with 5 ml of 10 % pneumonic lung suspension containing approximately 10^5 color changing units of M. hyopneumoniae. This pig-passaged lung inoculum was free of bacteria, viruses and mycoplasmas other than M. hyopneumoniae (6), when cultivated on aerobic blood agar, in cell culture, or in mycoplasma broth. The M. hyopneumoniae lung inoculum was prepared by grinding 1 gram of lung from a pig infected with a field strain of M. hyopneumoniae in 9 ml Friis broth medium (7) without added antibiotics. Control animals received 5 ml Friis mycoplasma broth, rather than lung homogenate, intratracheally. Previous work has indicated that lung

homogenate from non-infected pigs does not induce changes in the pig respiratory tract when inoculated intratracheally (8,9).

Necropsy. Six or 7 infected and 6 or 7 control pigs were necropsied at each of 3 intervals: 2, 4 and 6 weeks post-inoculation. Nasal, tracheal and bronchial secretions and lung homogenate (right middle lobe) from each pig were cultured for Bordetella bronchiseptica, Pasteurella multocida, Haemophilus spp., Actinobacillus pleuropneumoniae, and mycoplasmas similar to previously described methods (10).

Samples of the right cranial, right middle, and cranial and caudal segments of the left cranial lobe were collected from the lungs of most pigs. Occasionally the accessory lobe was substituted for the left cranial segment or the right cranial lobe. The tissues were fixed in formalin, paraffin embedded, prepared for sectioning and serial sections 5 um thick were processed for histochemical staining.

Histochemical Staining of Lung Tissue Sections. PAS/AB staining was performed according to described methods (3) and the sections were counterstained with hematoxylin. Modification of the previously published HID/AB staining procedure (3) was necessary to facilitate evaluation by image analysis. The staining time was extended to 36 hours for HID to intensify black color and shortened to 3 minutes for AB to diminish blue color without apparent effect on stain

specificity.

Image Analysis. Measurement of airway goblet cell mucin by image analysis was performed on 4 equidistant locations in mucosa of one bronchus in an HID/AB stained section of each lung lobe. The area of goblet cell mucin stained black or blue in each location was determined and quantitated in relationship to total epithelial area viewed in that image field (epithelium visible through the microscope; Figure 1A and B). The first area of epithelium evaluated was that part of the bronchus first visualized in the field. The second area measured was directly across the bronchus from the first, and the third and fourth areas were located in a line perpendicular to one transecting the first two locations.

The image analysis system used for this study consisted of 1) the camera^a, 2) the microscope^b, 3) the image processing system^c, composed of a microprocessor, array processor, digitizer tablet (magnetized) with camera, and 2 video monitors; one for software display and one for image display, and 4) the software program^d. All image fields were scaled so that the data output would represent the actual size of the objects of interest. Tissue sections were viewed through the microscope and input to the computer system. Images were input to the computer with and without a blue filter in the light path of the microscope which enabled measurement of black staining goblet cell mucin (sulphated

mucin) and total goblet cell mucin, respectively. Each image was stored in the computer from 0 (= black) to 255 (= white) in shades of gray. Stored images of HID/AB stained sulphated mucins and sialomucins were computer enhanced to allow the identification of black staining and blue staining structures.

The reference area was defined by creating a measuring frame around the perimeter of the epithelium in the image field. Discrimination allowed goblet cell mucin within the reference area to be selected for field area measurement. The field area of the image obtained without and with use of the blue filter represented total stained goblet cell mucin (Figure 1A) and black stained sulphated goblet cell mucin (Figure 1B), respectively. The percentage denoted by the ratio of field area to reference area was calculated for the images with and without a blue filter. Percentage area occupied by sialomucin (blue stain) was the difference between total and sulphated mucin percentage areas. Results were analyzed by analysis of variance with a 95 % confidence level ($p < 0.05$) and by linear regression.

Histology. Because it was not possible to consistently distinguish between colors created by PAS/AB stain with the image analysis system, these preparations were evaluated manually with light microscopy. Slides were evaluated without knowledge of pig identity to avoid subjective bias.

One randomly selected bronchus and one bronchiole were evaluated in each lobe. To avoid an airway size effect on uniformity of data, bronchi were required to have an inside diameter of 0.9-1.9 mm and bronchioles 0.15-0.25 mm, estimated with a micrometer. The number of epithelial cell nuclei between the basement membrane and the epithelial luminal surface within the randomly selected bronchus were counted, and the average was recorded as a score. At least 4 areas approximately 5 luminal cells in width were selected for counting. The first area counted was usually in the bronchial epithelium at the top of the field, the second was at the bottom, and the remaining 2 lay in a line perpendicular to one intersecting the first and second areas. More than 4 areas were evaluated in bronchi that exhibited large variations in epithelial nuclei number. The percent of total bronchial epithelial cells containing PAS and/or AB positive mucosubstance was estimated by counting epithelial cells with and without mucin in at least 4 areas of bronchial epithelium and subjective assessment of the entire epithelium. Areas were selected as described above, but were 10 to 40 luminal cells in width. A higher number of cells were evaluated when subjective assessment indicated that distribution of goblet cells within the epithelium was not uniform. All cells in bronchioles were counted because total cell numbers were less than 200 for most bronchioles. The

percent of total cells staining preferentially with PAS or AB in submucosal glands around the selected bronchus was estimated by counting 20-50 epithelial cells in 2-4 representative areas, depending on availability, and subjective assessment of the entire gland. Mucous and serous cells were not differentiated. Descriptive data were converted to numerical scores and all data were evaluated statistically by analysis of variance with a 95 % confidence level ($p < 0.05$).

RESULTS

Culture/serology. Nasal, tracheal and bronchial secretions and lungs from all pigs were culture negative for Bordetella bronchiseptica, Pasteurella multocida, Hemophilus spp. and Actinobacillus pleuropneumoniae. Mycoplasma hyopneumoniae was isolated from lungs of all infected pigs, but not from any control pigs. All pigs were negative for CF antibody to M. hyopneumoniae before inoculation. Infected pigs were CF positive 2 weeks after inoculation until necropsy.

Bronchi. Goblet cells of infected bronchi in sections stained with PAS/AB were slender and empty when compared to the typical chalice shape of goblet cells in the bronchi of control pigs (Figure 2A and B). A lower percentage of bronchial epithelial cells in pigs infected with M. hyopneumoniae contained acid mucosubstance (AB +) than age matched control pigs at all time intervals ($p < 0.0001$; Figure 3). Neutral mucosubstance (PAS +) was decreased in bronchial cells of infected pigs at all time intervals, but was significantly decreased ($p < 0.04$) in infected pig airways only at 4 weeks after infection when compared to appropriate control pigs (Figure 3). However, the ratio of PAS to AB staining cells in bronchi of infected pigs was not significantly different than control pigs throughout the present experiment. In control and infected pigs a single

goblet cell occasionally contained both neutral and acid glycoprotein. Goblet cells staining exclusively for neutral mucin were rarely observed. The number of epithelial cell nuclei between the basement membrane and the epithelial luminal surface was greater in infected pigs than in control pigs throughout the experiment, particularly at 2 and 4 weeks post infection (data not shown).

Image analysis of sections stained with HID/AB revealed that goblet cells of pigs inoculated with M. hyopneumoniae contained a significantly ($p < 0.05$) greater area (measured as percent epithelial area) with sulphated mucin than control pigs, even though the area of total mucin (sulfomucin and sialomucin) was significantly less ($p < 0.05$) in the former group (Figure 4). The respective area occupied by sulphated mucin, sialomucin and total mucin was similar at all 3 time intervals in pigs infected with M. hyopneumoniae, but increased in a linear fashion in control pigs as they became older ($r = .98$; Figure 4).

Submucosal glands. In PAS/AB stained sections, the percent of bronchial submucosal gland epithelial cells preferentially staining for acidic mucosubstance (AB+) in infected pigs was not significantly different than control pigs, even though glands of infected pigs had a larger percentage of AB+ cells (Figure 5). In contrast, bronchial submucosal glands of infected pigs contained a significantly

lower percentage ($p < 0.04$) of cells preferentially stained for neutral mucosubstance (PAS+) than control pigs 6 weeks after infection (Figure 5).

Bronchioles. In pigs infected with M. hyopneumoniae the total percent of bronchiolar epithelial cells containing stained mucosubstance was significantly less than in control pigs at all intervals ($p < 0.03$; Figure 6). Likewise, the percent of bronchiolar epithelial cells in M. hyopneumoniae infected pigs staining for neutral mucosubstance was significantly lower than age-matched control pigs throughout the experiment ($p < 0.0001$; Figure 6). Although a higher percentage of bronchiolar cells from infected pigs contained acidic mucin throughout the experiment, the percent of bronchiolar cells staining with AB was not significantly different from control pigs, probably because of wide variability in values within groups (Figure 6).

DISCUSSION

Acidic mucin and sulphomucin were significantly increased ($p < 0.05$) and neutral mucosubstance was decreased in the mucosal goblet cells of pigs infected with M. hyopneumoniae when compared to appropriate control pigs. These results contrast with a previous report (11) in which neutral mucins predominated over acidic mucins, such as sulphomucins, in the bronchial goblet cells of pigs infected with porcine mycoplasma, and acidic mucins predominated in control pigs. However, sulphated mucins in the previous study (11) were identified with PAS/AB stain at pH 1.0; only sulphated mucosubstances stain with AB at pH 1.0 (3). Sulphated mucins in the present study were identified by staining with HID/AB. The age-related linear increase in sulphated mucin of control pigs correlated well with results reported previously (12).

Goblet cells of infected bronchi were slender and empty when compared to the typical chalice shape of goblet cells in the bronchi of control pigs (Figure 2). Similar changes in airway goblet cell shape and staining patterns were previously reported in pigs infected with porcine mycoplasma (11). Identification of neutral mucosubstance in bronchiolar epithelium was more difficult than in bronchi because of the presence of other PAS+ material within some bronchiolar cells. Presence of D-galactose, L-fucose, N-

acetylglucosamine or N-acetylgalactosamine in tissue components other than epithelial mucins may cause positive staining reactions (3).

The increased number of epithelial cell nuclei between the basement membrane and the epithelial luminal surface of infected pigs when compared to control pigs throughout the experiment agrees with hyperplasia of bronchial epithelium in pigs observed by others as early as 7 days after inoculation with porcine mycoplasma (9,11). Porcine tracheal organ cultures also were reported to show evidence of thickening of the epithelium following inoculation with M. hyopneumoniae (13). Lymphohistocytic peribronchial cuffing accompanied by increased numbers of leukocytes within bronchial epithelium of infected pigs (data not shown) probably reflected activation of the immune response and supported established histologic descriptions of the disease (1,2).

Increased sulphomucin in the goblet cells of pigs infected with M. hyopneumoniae implied that the cells had switched to increased sulphation of mucosubstance. Release of sulphated glycoconjugates from the surface of ciliated cells may be increased in response to proteinases generated by bacteria or host cells during infection (14). However, the present study did not exclude the possibility that sulphomucin and sialomucin were released at different rates after synthesis, leading to a quantitative increase of

sulphomucin in goblet cells of infected pigs.

Ciliostasis occurring during infection with M. hyopneumoniae may contribute to increased mucopurulent exudate in small airways even when mucin secretion is normal or decreased. Increased HID staining of goblet cells in infected pigs may reflect hypersecretion in response to M. hyopneumoniae infection because sulphation increases with hypersecretion in other models (15). Mucopurulent exudate observed in airways of pigs with M. hyopneumoniae infection may be due to hypersecretion of mucus as well as ciliostasis.

Increased secretion of sulfomucin may also be induced in response to local irritation (15,16). Mycoplasma hyopneumoniae produces hydrogen peroxide^e which could precipitate local irritation of mucosal cells. Complement activation, neutrophil degranulation and other inflammatory mediators released during the host immune response to M. hyopneumoniae probably contribute to irritation of the mucosal surface. Furthermore, some inflammatory mediators (basic polypeptides such as kinins) have been shown to modulate glycoprotein synthesis in canine tracheal explants (17).

Sulphation of mucin molecules, which occurs post-translationally in the Golgi apparatus, may or may not affect rheology of the mucous blanket. Marriott and Irons (18) found no correlation between elasticity or viscosity and

sulphate content of salivary mucus, even though they demonstrated that a sulphated glycoprotein was involved in gel formation. However, Mian et al. (19) demonstrated that N-acetylneuraminic acid content correlated well with low viscosity and sulphate ester content with high viscosity of mucins. An increase in the highly sulphated component of mucin has been correlated with increased viscosity of tracheobronchial mucus secretion in cystic fibrosis patients (20).

Factors other than sulphation of glycoproteins may confound attempts to correlate sulphated component content with altered rheology of mucus. Alterations in pH, ionic strength and glycoprotein, protein, DNA, chloride or calcium concentration can produce significant changes in viscoelastic behavior of tracheal mucus (21,22,23,24,25).

Sulphation of mucus may increase the lifetime of some species of glycoproteins (26,27). Adherence to receptors in the mucus layer may enhance colonization of mucosal surfaces by microorganisms (28), possibly through increased longevity of glycoproteins. Changes in mucus secretion might contribute to the enhanced susceptibility to secondary bacterial respiratory infections often observed in swine infected with M. hyopneumoniae. Additionally, penetration of bacteria to the mucosal cell surface may be augmented by planes of stress in the mucus layer created during extrusion

of goblet cell contents (28), which is presumably more frequent during hypersecretion. Increased acidic component of mucosubstance in goblet cells and submucosal glands of infected pigs when compared to age matched control pigs implied that the glycoprotein component of the mucociliary blanket of infected pigs contained more anionic sites than did the glycoprotein component of control pigs. The effect of changes in goblet cell mucin carbohydrate on M. hyopneumoniae colonization or survival is unknown.

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FOOTNOTES

- a CCD Video black/white camera, Panasonic
- b Axiophot model, Zeiss
- c Zeiss
- d IBAS Measuring Program, copyright Kontron Elektronik
- e DeBey MC, Ross RF. Hydrogen peroxide production by Mycoplasma hyopneumoniae. Proc Conf Res Workers An Dis 1988;69:68 (abstr).

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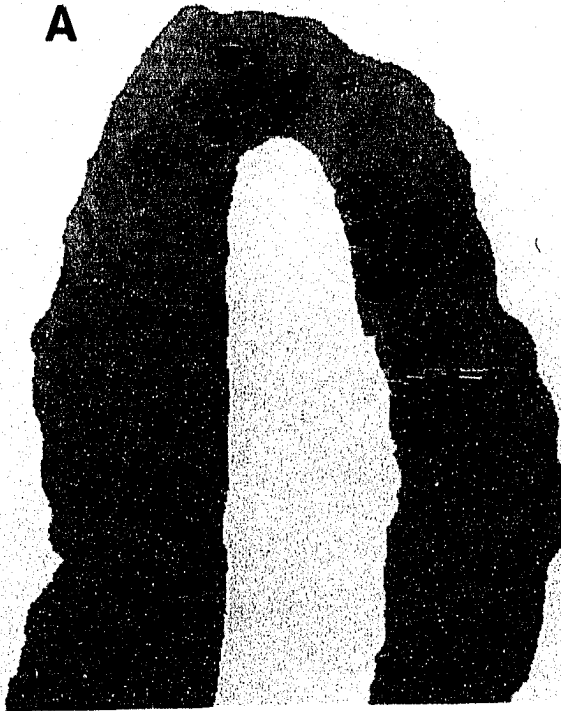
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Figure 1. Computer enhanced image of bronchial epithelium from a pig 6 weeks after infection with M. hyopneumoniae generated to demonstrate total (A) or sulphated (B) goblet cell mucin.

A



B

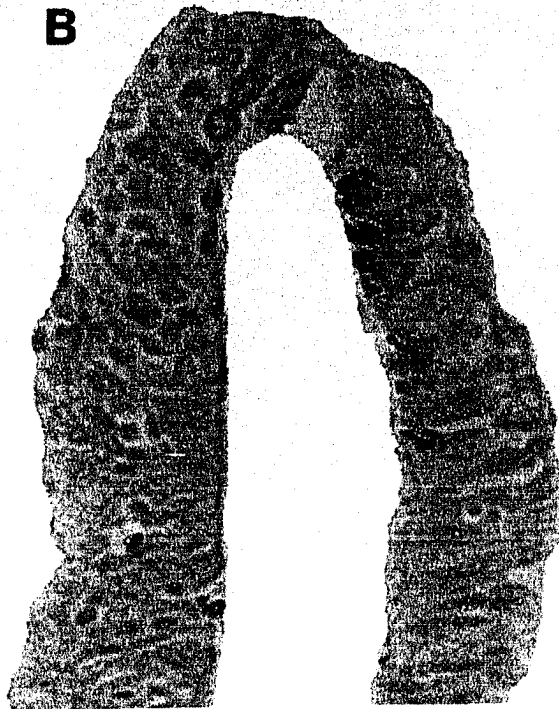
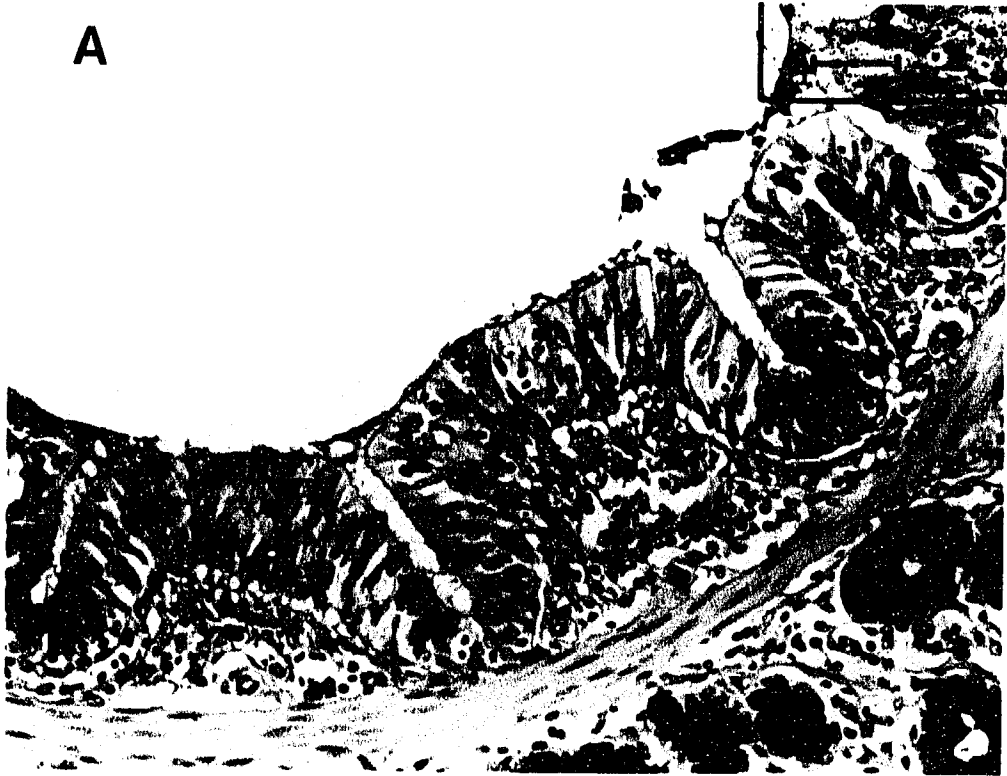


Figure 2. A. Bronchial epithelium from a pig 6 weeks after inoculation with M. hyopneumoniae stained with Periodic acid-Schiff/Alcian Blue followed by hematoxylin (100x; bar = 39um). Clumping or patchy loss of cilia is apparent. Cellularity of the submucosa and intra-epithelial leukocytes are increased in the bronchial epithelium when compared to a bronchus of similar size in an age matched control pig (B).

A



B



Figure 3. Percent of epithelial goblet cells containing neutral or acidic mucin in bronchi of control pigs or pigs infected with M. hyopneumoniae. Periodic acid-Schiff/Alcian Blue stain (PAS/AB). Bronchi of infected pigs contained a significantly lower* percent total cells with mucin or acidic mucin ($p < 0.0001$) at all intervals post infection (PI), and significantly less neutral mucin 4 weeks PI than control pigs ($p < 0.037$).

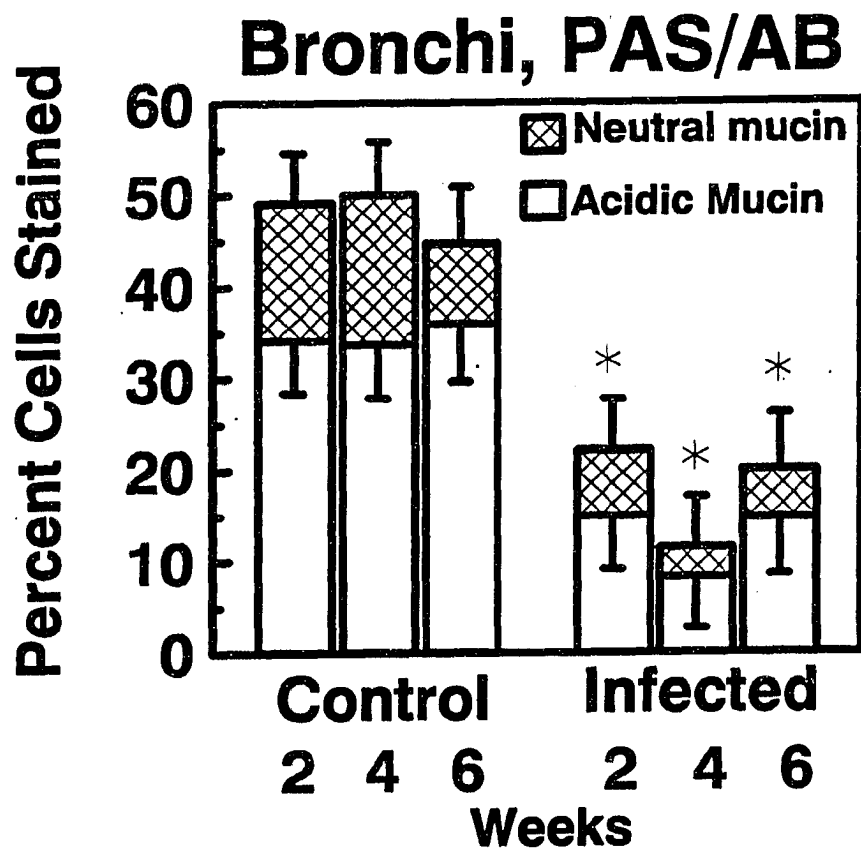


Figure 4. Percent of bronchial epithelium occupied by goblet cell mucin in control pigs and in pigs infected with M. hyopneumoniae. High iron diamine/Alcian blue stain (HID/AB). Bronchi of infected pigs contained significantly less* total mucin and sialomucin, and significantly more sulphomucin than bronchi of control pigs ($p < 0.05$).

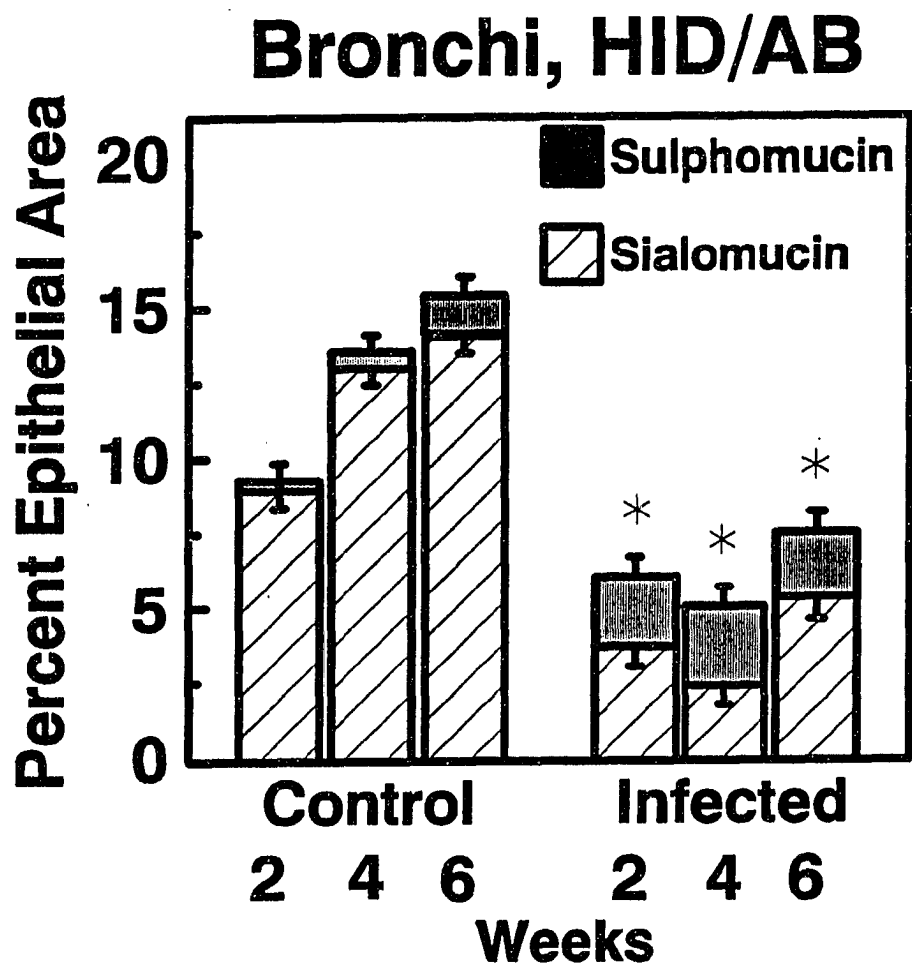


Figure 5. Percent of bronchial submucosal gland epithelial cells preferentially staining for neutral or acidic mucin in control pigs, or in pigs after infection with M. hyopneumoniae. Periodic acid-Schiff/Alcian Blue stain. Neutral mucin was significantly decreased* in submucosal glands around infected bronchi 6 weeks post-infection ($p < 0.042$).

Submucosal Glands, PAS/AB

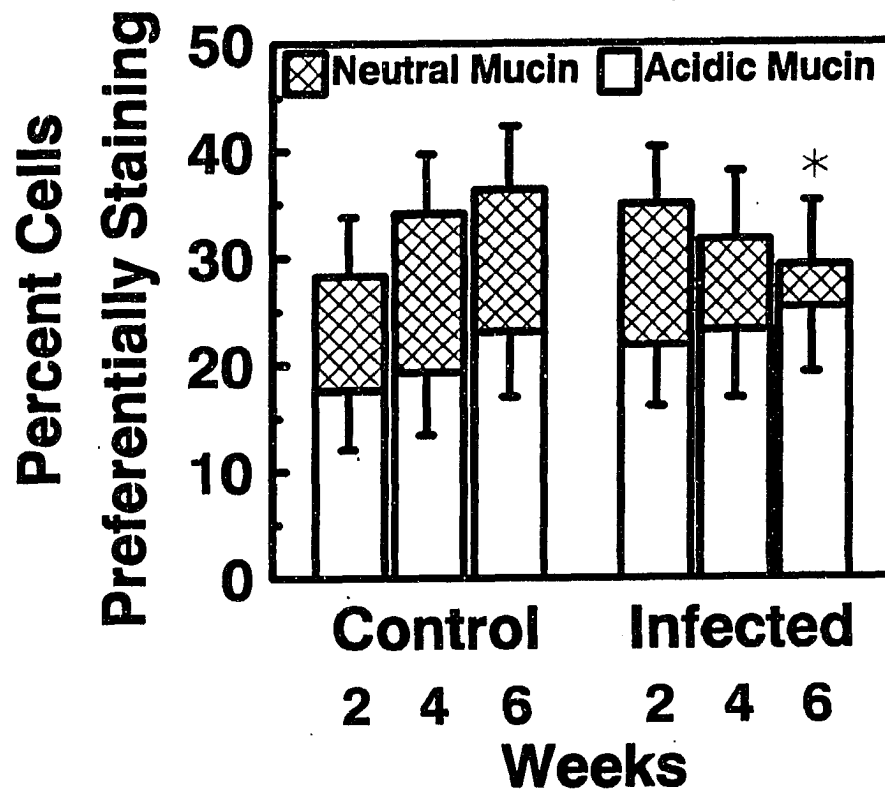
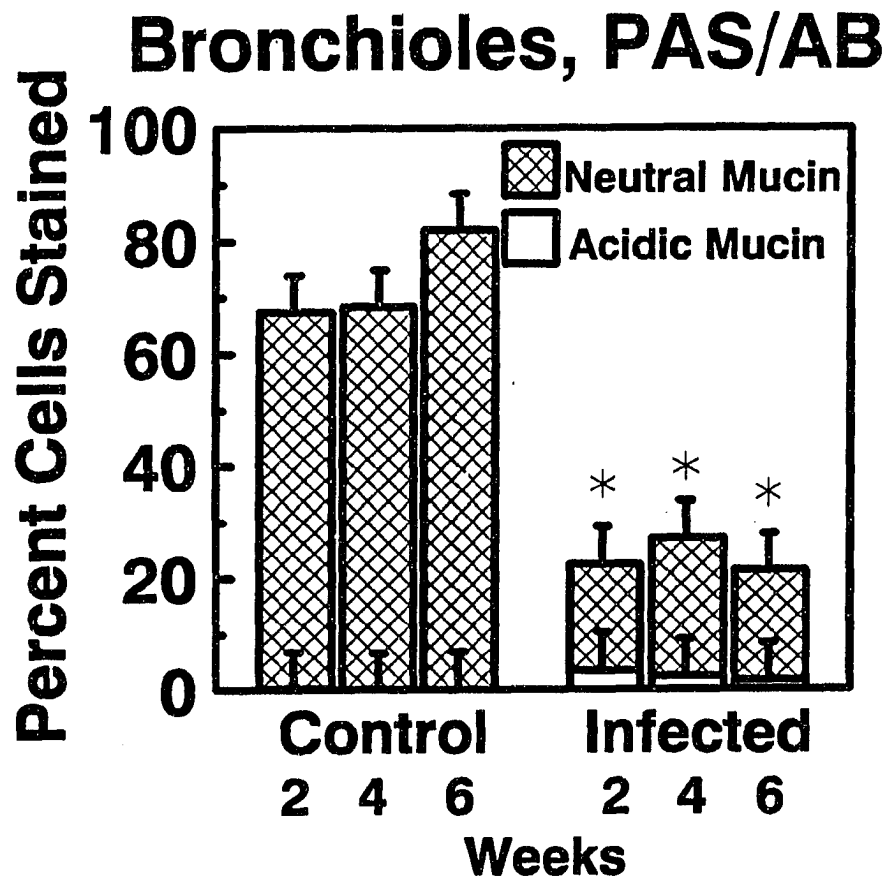


Figure 6. Percent of bronchiolar epithelial cells containing neutral or acidic material in control pigs, or in pigs infected with M. hyopneumoniae. Periodic acid-Schiff/Alcian Blue stain. At all intervals percent of cells in bronchioles from infected pigs containing stained substance ($p < 0.026$) or PAS+, neutral material ($p < 0.0001$) was significantly less* than control pigs.



SECTION II: CILIOSTASIS AND LOSS OF CILIA INDUCED
BY MYCOPLASMA HYOPNEUMONIAE IN
PORCINE TRACHEAL ORGAN CULTURES

Ciliostasis and Loss of Cilia Induced by Mycoplasma
hyopneumoniae in Porcine Tracheal Organ Cultures

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ABSTRACT

In vivo and in vitro grown Mycoplasma hyopneumoniae was inoculated onto newborn piglet tracheal organ cultures to study mechanisms utilized by this organism to damage ciliated respiratory epithelium. Ciliostasis and loss of cilia induced by M. hyopneumoniae was evaluated with an inverted microscope by estimating ciliary retention and activity, or by light microscopic examination of fixed, sectioned tracheal rings. Association of M. hyopneumoniae with ciliated epithelium was assessed using immunofluorescence and/or electron microscopy. Epithelial damage induced by M. hyopneumoniae in tracheal ring epithelium diminished with in vitro passage of the organism. Higher passage cultures were not induced to cause ciliostasis or loss of cilia when grown in mycoplasma medium supplemented with porcine lung extract. Levels of dehydrogenase enzymes or calmodulin in tracheal ring epithelium were not altered even though ciliostasis and loss of cilia induced by M. hyopneumoniae was extensive. Epithelial damage by M. hyopneumoniae in tracheal rings was averted by porcine immune serum or by separating organisms from ciliated epithelium with a 0.1 μ m membrane. Attachment of M. hyopneumoniae to ciliated epithelium was necessary to induce ciliostasis and loss of cilia in this model.

INTRODUCTION

Mycoplasma hyopneumoniae causes enzootic pneumonia in swine. Electron microscopic examination of lung tissue from swine infected with M. hyopneumoniae has demonstrated a close association of the organism with ciliated epithelial cells of the trachea and bronchi in infected pigs (Mebus and Underdahl, 1977; Underdahl et al., 1980; Tajima and Yagihashi, 1982).

Damage to ciliated epithelium induced by M. hyopneumoniae in vivo is evident in histologic sections of infected lungs as loss or clumping of cilia in affected airways (Livingston et al., 1972; Underdahl et al., 1980; DeBey et al., in press). Progressive ciliostasis and epithelial exfoliation in porcine tracheal rings co-cultivated with fetal porcine lung fibroblasts and M. hyopneumoniae were reported by Williams and Gallagher (1978). However, most workers observed that M. hyopneumoniae caused no ciliostasis or loss of cilia in vitro (Pijoan et al., 1972; Cherry and Taylor-Robinson, 1973; Rosengarten and Kirchhoff, 1981).

In vitro growth for as few as 6 passages led to loss of pathogenicity of M. hyopneumoniae for pigs (Tajima and Yagihashi, 1982). Zielinski and Ross (1990) reported that a highly passaged strain of M. hyopneumoniae failed to induce pneumonia in pigs, while strains passaged fewer times caused

lesions. Loss of virulence in vitro might be due to loss of stimuli recognized by M. hyopneumoniae in vivo when associated with the respiratory mucosa of a pig.

Assessment of mycoplasmal effects by measuring enzymatic activity or host cell proteins in inoculated and uninoculated organ cultures has been used to quantitate epithelial damage induced by other mycoplasmas. Dehydrogenase enzyme levels in hamster tracheal rings inoculated with M. pneumoniae and calmodulin content in equine oviduct rings inoculated with M. equigenitalium were lower than values obtained with corresponding controls (Gabridge and Polisky, 1976; Bermudez et al., 1990).

In this study we used porcine tracheal organ cultures to establish an in vitro model for detection of M. hyopneumoniae-induced damage to ciliated respiratory epithelium. Epithelial damage by M. hyopneumoniae to tracheal ring epithelium diminished with in vitro passage, and was averted by preventing direct contact of organisms with cilia.

MATERIALS AND METHODS

Lung homogenate and M. hyopneumoniae. Lung homogenate (LH) containing M. hyopneumoniae was obtained from an SPF (specific pathogen free) pig infected with M. hyopneumoniae strain 232, a derivative of strain 11 (Mare and Switzer, 1965; Bereiter et al., 1990). The homogenate contained 10% (w/w) lung tissue in Friis mycoplasma medium (Friis, 1975) without antibacterials, and served as a source of in vivo grown M. hyopneumoniae (Mh). The Mh was examined for mycoplasmas or other bacteria by culturing in mycoplasma medium (Ross and Whittlestone, 1983) and on blood agar, and for viruses by transmission electron microscopy (TEM) and immune TEM. Supernate of Mh was prepared by heating at 100 C for 2 minutes and centrifugation at 740 x g for 15 minutes (heated Mh). Filtrate (Fil-10) and retentate (Ret-10) were prepared by passing heated Mh through a filter retaining molecules greater than approximately 10,000 molecular weight (Centricon-10 Microconcentrator, Amicon, Division of W.R. Grace and Co., Danvers, MA). A portion of Mh was subjected to 180,000 rad (Ir Mh). Supernate of Ir Mh was prepared by centrifugation at 740 x g for 15 minutes (Cent-Irrad). Lung homogenate collected from a normal SPF pig, free of infection with M. hyopneumoniae, was prepared as a 10% (w/w) suspension in Friis mycoplasma broth and designated NLH. Aliquots of lung preparations were stored at -70 C until used.

The Mh was diluted tenfold in Friis mycoplasma broth, incubated, passaged in Friis broth and stored at -70 C to provide second, third, fourth, fifth, tenth and twentieth passage M. hyopneumoniae. Strain 232, used for inoculation of a pig to produce Mh, was cloned (clone 2A3; 232C), grown to passage 40 in Friis broth and frozen at -70 C. At passage 36 232C induced pneumonic lesions in pigs inoculated intratracheally (Bereiter et al., 1990). No ciliostasis or loss of cilia was detected after inoculation of porcine tracheal rings with Strain 232C in preliminary experiments (See Appendix A). For inoculation to tracheal rings, frozen cultures were thawed and diluted in Friis mycoplasma broth without thallium acetate.

Quantitation of M. hyopneumoniae. Numbers of organisms inoculated onto tracheal rings were estimated by the tube dilution method for determination of color changing units per ml (CCU) for every experiment. One CCU represented the highest dilution of Mh or culture changing the color of a tube of Friis medium from red to yellow.

Tracheal Rings. Crossbred Hampshire and Yorkshire piglets were obtained from sows from commercial swine herds by ceasarean section or by manual removal of the piglet from the birth canal during normal parturition. Newborn piglets were immediately placed into a sterile transport container. Within 1-5 hours of birth, each piglet was deeply

anesthetized with approximately 65 mg sodium pentobarbital given intraperitoneally and the trachea aseptically removed at the level of the right bronchus as described for hamsters (Collier and Carson, 1983). The excised trachea was placed into approximately 5.0 ml Friis medium without thallium acetate and cut into 20-25 rings. Each ring was randomly assigned to a well in a 24 well tissue culture plate (Costar Tissue Culture Cluster²⁴, #3424, 205 Broadway, Cambridge, MA). Rings were incubated with or without M. hyopneumoniae in Friis medium without thallium acetate at 35.5 C in 0.5% CO₂.

Direct evaluation of damage to ciliated epithelium.

Cytotoxicity of M. hyopneumoniae for tracheal ring epithelium, manifest as loss of cilia from epithelial cells and ciliostasis, was evaluated using an inverted microscope (100X) with slight modification of the methods described by Gabridge and Polisky (1976). Loss of cilia was determined after estimating the percent of epithelium retaining ciliated cells (0 to 100%). Ciliostasis, evaluated by observing the vigor of ciliary beating, was scored on a scale from 0 (no movement) to 3 (vigorous beating). Because loss of cilia and ciliostasis usually occurred simultaneously, only ciliary loss data is presented. For ciliostasis data, see Appendix A.

Immunofluorescence (FA). Tracheal rings were frozen at -70 C in embedding medium for frozen tissue sections (O.C.T. Compound, Miles Inc., Diagnostics Division, Elkhart, IN), sectioned and stained with fluorescein labelled porcine origin antibody to M. hyopneumoniae. Stained sections of tracheal rings and routine positive control sections were examined for the presence of immunofluorescence specific for M. hyopneumoniae (Amanfu et al., 1984).

Histopathology. Selected tracheal rings were fixed in 10% buffered neutral formalin, embedded in paraffin, cut at 5 um, processed and stained with hematoxylin and eosin by standard methods.

Electron Microscopy. Selected tracheal rings were fixed at 4 C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (CB), pH 7.2, postfixed in 1% osmium tetroxide in CB for 2 hours at room temperature, washed with distilled water, dehydrated in a graded series of acetone of 50, 75, 95 and 100% and embedded in Embed 812 resin (Electron Microscopy Sciences, Fort Washington, PA). Thick sections were cut at 1-2 um with glass knives and stained with 1% Toluidine blue. Thin sections were cut at 70-90 nm with a diamond knife, stained with 2% uranyl acetate and Reynold's lead citrate and examined in a Hitachi H500 transmission electron microscope at 75 KV.

Experimental design. Appropriate control or mycoplasma preparation in 0.3 ml Friis medium was randomly assigned to each well of a 24 well tissue culture plate. At least 10 tracheal rings were used for each control or mycoplasma preparation. Spent medium was removed daily and 0.3 ml fresh Friis medium added to each well until the ring was removed or the experiment was terminated. The medium from each well containing a ring was cultured for M. hyopneumoniae and other bacteria whenever a ring was removed.

Effect of lung constituents on cytotoxicity of M.

hyopneumoniae. For inoculation to tracheal rings, Mh was diluted 1:10, 1:100, 1:1000, and 1:100,000 in Friis mycoplasma medium. The Mh, Ir Mh and NLH were diluted 1:10 in Friis mycoplasma medium without thallium acetate. The Ir Mh, Cent-Irrad, NLH, heated Mh, Fil-10 or Ret-10 were diluted 1:10 in Friis medium with or without passage 2 of M. hyopneumoniae for inoculation to tracheal rings.

Effect of in vitro passage on cytotoxicity of M.

hyopneumoniae. Passages 2, 3, 4, 5, 10, and 20 of M. hyopneumoniae were assessed for ability to induce ciliary loss. Ten percent suspensions of Mh were propagated in Friis medium from passage 2 to passage 10 with or without 10% NLH or heated Mh. Strain 232C was diluted 1:10 in Friis mycoplasma medium with or without Ir Mh for inoculation to tracheal rings.

Effect of antibody on cytotoxicity of M. hyopneumoniae.

Convalescent serum was obtained from 2 pigs 4 weeks after intratracheal challenge with in vivo grown M. hyopneumoniae. Convalescent sera (CSS) contained antibodies specific for M. hyopneumoniae detected by the complement fixation test

(Slavik and Switzer, 1972). Normal serum (NSS) without antibodies specific for M. hyopneumoniae by ELISA (Bereiter et al., 1990) was obtained from pigs in an SPF, barrier-maintained herd known to be free of M. hyopneumoniae.

Positive control tracheal rings were inoculated on Day 0 with LH from a pig infected with M. hyopneumoniae in Friis mycoplasma medium (diluted 1:10). On subsequent days spent medium was replaced with Friis mycoplasma medium. Other rings received an initial inoculum on Day 0 of LH from a pig infected with M. hyopneumoniae (1:10) and NSS or CSS (1:2) in Friis mycoplasma medium. On subsequent days wells were replenished with appropriate serum in Friis mycoplasma medium (1:4).

Inhibition of M. hyopneumoniae-induced ciliary loss with a 0.1 μ m membrane. Sterile Millicell inserts containing a membrane of 0.1 μ m pore size (Millicell, Custom Order No. SD2P111E1, Millipore Corporation, Bedford, MA) and designed for 24 well plates were placed in each well of a plate. Tracheal rings were placed on top of the inserts. Spent medium under the membrane in each well was removed daily and

fresh Friis mycoplasma medium added until the experiment was terminated.

Control rings were inoculated with sterile Friis mycoplasma broth on top of and below the Millicell insert membrane. Positive control rings were inoculated with M. hyopneumoniae on top of (direct exposure) and below the Millicell membrane, to insure direct contact of ciliated epithelium with viable mycoplasma organisms. Test rings were inoculated with Friis medium on top of the Millicell membrane and M. hyopneumoniae was placed below the membrane (indirect exposure). All tracheal rings receiving M. hyopneumoniae either by direct or indirect exposure were inoculated with the same total number of organisms, approximately 10^6 CCU M. hyopneumoniae. The medium from above and below the Millicell membrane in each well containing a ring was cultured for M. hyopneumoniae and other bacteria when the experiment was terminated. Colonization of tracheal rings by M. hyopneumoniae was evaluated by immunofluorescence.

Evaluation of dehydrogenase enzyme levels in porcine tracheal rings. Reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) by dehydrogenase enzymes of metabolically active cells of tracheal rings was measured as described previously (Gabridge and Polisky, 1976) with modifications. Rings with and without M. hyopneumoniae were evaluated. After incubation with TTC, the ring was placed in a vial containing

1.0 ml methanol for 30 minutes to extract formazan dye resulting from reduction of TTC. Triplicate 200 ul aliquots of methanol from each vial were assigned to a 96 well plate and the optical density (OD) was determined using an ELISA plate reader with a 490 nm filter. Dry weight of each tracheal ring was determined and OD/mg dry weight was calculated.

Evaluation of calmodulin levels in tracheal ring epithelium.

Two or five days after inoculation, epithelium from each tracheal ring was dissected from the cartilage, weighed and homogenized. Calmodulin and protein content of heated (100C) tissue homogenate were measured after centrifugation at 740 and 105,000 x g to assess total or unbound intracellular calmodulin, respectively. Volume of spent medium removed daily from each well was recorded and pooled by well for measurement of extracellular calmodulin content. Pooled medium from each well was cultured for M. hyopneumoniae and bacteria, heated (100C), centrifuged (105,000 x g) and evaluated for calmodulin and protein content.

Calmodulin levels of triplicate samples were assessed indirectly by measuring phosphate release from AMP in a calmodulin activated enzyme cascade described by Sharma and Wang (1979) with modifications (Bermudez, 1990; Bermudez et al., 1990). Cellular calmodulin content was calculated with reference to protein content (BCA Protein Assay, Pierce,

Rockford, IL) and explant tissue weight. Extracellular calmodulin content was calculated with reference to protein content and total volume of medium.

Statistical Analysis. Effects of M. hyopneumoniae on tracheal rings were determined with analysis of variance using an F-statistic ($p < 0.05$), and using the student's T-test to locate significant differences. The relationship between presence of M. hyopneumoniae and calmodulin level was determined with Pearson correlation coefficients ($r > 0.8$).

RESULTS

Lung homogenate and M. hyopneumoniae. The Mh contained 10^7 CCU of M. hyopneumoniae. Mycoplasma hyopneumoniae was the only organism isolated from Mh. No viral agents were detected by TEM or immune TEM, and mycoplasma were the only bacteria present. No mycoplasma or pathogenic bacteria were recovered from Ir Mh or NLH.

In experiments where ciliary loss was significant, the inoculum on Day 0 contained 10^5 to 10^8 CCU of passage 2 M. hyopneumoniae. Inocula containing Mh (1:10) possessed 10^5 to 10^6 CCU of M. hyopneumoniae. Medium bathing rings inoculated with Mh contained 10^6 CCU of M. hyopneumoniae 1 day post inoculation (PI), and approximately 10^8 CCU thereafter to 7 days PI. The CCU of passage 2 M. hyopneumoniae in spent medium at the termination of experiments was usually 10^8 .

Effect of lung constituents on cytotoxicity of M.

hyopneumoniae. In vivo grown and passage 2 M. hyopneumoniae caused significant ciliostasis and loss of cilia ($P < 0.04$; Figures 1 and 2) when compared to controls 4 and 5 days after inoculation of tracheal rings. Ciliostasis was closely associated with loss of cilia (See Appendix A).

Immunofluorescence specific for M. hyopneumoniae was evident in inoculated rings from 1 to 8 days PI. Specific immunofluorescence was not observed on a few rings exhibiting severe loss of cilia, ie. retaining less than 20% ciliated

epithelium on the luminal surface.

Microcolonies of organisms closely associated with ciliated epithelium were detected using TEM 2 days after inoculation. Extensive colonization of almost all intact ciliated epithelium was evident 5 days after inoculation with 10^5 CCU or greater of Mh (Figure 3) or passage 2 of Mh.

Examination of tracheal ring sections using light microscopy and TEM revealed that ciliated epithelium was replaced by a single layer of low cuboidal to flat epithelial cells where the luminal epithelial surface had been damaged. Injury leading to loss of ciliated epithelium occurred during ring preparation or in response to damage induced by mycoplasmas. Cilia from control rings usually appeared straight and separated from one another when viewed by light microscopy. In contrast, cilia of inoculated rings viewed by light microscopy were attached to one another at the tips forming ciliary clumps up to 30 μ m wide. Basophilic, spherical organisms (mycoplasmas) approximately 0.3 μ m in diameter and adherent to cilia were visible in areas of ciliary clumping.

Tracheal rings inoculated with 10^5 CCU or greater of Mh (1:10 dilution) exhibited significant loss of cilia 5 days PI, whereas onset of ciliary loss was delayed by further dilution of mycoplasma inoculum. Time required for induction of epithelial damage by in vivo grown M. hyopneumoniae was

directly related to dose (Figure 4). Colonization of tracheal ring epithelium was evidenced by positive immunofluorescence of almost all rings inoculated with dilutions of Mh upon termination of the experiment. In experiments where the inoculum of passage 2 contained 10^3 or fewer CCU, no significant ciliostasis was observed and no immunofluorescence specific for M. hyopneumoniae was observed.

Effect of in vitro passage on cytotoxicity of M.

hyopneumoniae. Tracheal rings were inoculated with 10^5 or greater CCU of each M. hyopneumoniae passage. At termination of the experiment 5 days PI, 10^6 to 10^8 CCU of M. hyopneumoniae were recovered from spent medium.

Passage 2 of Mh consistently induced more ciliary loss in piglet tracheal rings than higher in vitro passages. A single experiment including in vitro passages 2 to 20 of Mh is shown in Figure 5, and in this experiment passage 20 induced significant ciliary loss when compared to uninoculated controls ($P < 0.01$). However, induction of significant epithelial damage by passage 20 of Mh was not consistent in other experiments, while passage 40 or greater of 232C was consistently noncytotoxic.

Effect of lung preparations on cytotoxicity of M.

hyopneumoniae. In vitro growth in the presence of heated Mh or NLH did not enhance the cytotoxicity of M. hyopneumoniae when compared to organisms simultaneously passaged without lung homogenate. Clear evidence of an effect of lung preparations on ciliary loss induced by passage 2 of M. hyopneumoniae at 5 days PI was not found (See Appendix A for detailed data).

Epithelial damage induced by strain 232C (passage 40) in porcine tracheal rings was not enhanced by addition of Ir Mh. For detailed data, see Appendix A. The CCU of M. hyopneumoniae 232C recovered from medium at the end of the experiment were 100-10,000 times greater than CCU inoculated. Rings inoculated with 232C had only rare focal areas of specific immunofluorescence on the epithelium.

Effect of antibody on cytotoxicity of M. hyopneumoniae. The CSS significantly mitigated cytotoxicity of M. hyopneumoniae to ciliated cells for 3 to 5 days PI ($P < 0.03$; Figure 6).

The CCU of M. hyopneumoniae recovered from medium containing CSS were 1000 times less than CCU from medium containing NSS. Colonization of tracheal epithelium of NSS- and CSS-treated rings was evident 5 days PI when examined by FA and TEM.

Inhibition of M. hyopneumoniae-induced ciliary loss with a 0.1 μ m membrane. Separation of M. hyopneumoniae from ciliated epithelium by a 0.1 μ m membrane prevented loss of

cilia ($P < 0.01$; Figure 7). At termination of the experiment 5 days PI, 10^7 to 10^9 CCU M. hyopneumoniae were recovered from media below the Millicell membrane in wells inoculated with the organism. Mycoplasmas were not usually recovered from above the Millicell membrane of rings indirectly exposed to M. hyopneumoniae. When recovered, CCU of mycoplasma in medium above the Millicell membrane (10^5) were 1000 times less than CCU recovered below the membrane (10^8) in the same well. No mycoplasmas were recovered from negative control wells.

Metabolic Assays. Total and unbound intracellular calmodulin levels (ug calmodulin/mg protein) of rings inoculated with M. hyopneumoniae (M) were not significantly different from controls (C) 2 days (Total M=3.4, C=4.9; Unbound M=5.0, C=7.2) or 5 days (Total M=14.3, C=13.5; Unbound M=5.9, C=5.7) after inoculation. Likewise, extracellular calmodulin levels were not significantly different 2 days (M=3.7, C=4.6) or 5 days (M=1.8, C=1.5) after inoculation. Total and unbound intracellular calmodulin levels (ug calmodulin/mg wet weight) were not significantly different 2 days (Total M=556.9, C=703.9; Unbound M=203.3, C=324.3) or 5 days (Total M=913.1, C=794.0; Unbound M=191.9, C=179.3) after inoculation. Likewise, extracellular calmodulin levels (ug/ml medium) were not significantly different 2 days (M=9.1, C=7.8) or 5 days (M=2.2, C=2.6) after inoculation. There was no relationship

between calmodulin level and loss of ciliated epithelium or ciliary activity. Mean dehydrogenase enzyme levels, expressed as optical density/mg dry weight, were not significantly affected by M. hyopneumoniae 5 days after inoculation (M=.170, C=.173).

DISCUSSION

In vivo grown or passage 2 M. hyopneumoniae consistently induced marked loss of cilia and reduction in the ciliary activity of porcine tracheal rings. Positive immunofluorescence and TEM visualization provided evidence that numerous M. hyopneumoniae organisms were closely associated with ciliated epithelium of tracheal organ cultures exhibiting ciliostasis and loss of cilia. Large numbers of organisms were recovered after inoculation with viable Mh, in vitro passages of Mh, and 232C (passage 40) indicating that growth in the presence of tracheal rings did not correspond with cytotoxic potential.

Close association of M. hyopneumoniae with ciliated cells was essential for induction of epithelial damage in this model. Tracheal rings indirectly exposed to M. hyopneumoniae through a 0.1 um membrane failed to exhibit loss of cilia when compared to rings directly inoculated with the organism. Mycoplasma hyopneumoniae apparently does not secrete a diffusible substance cytotoxic for ciliated epithelium in vitro. However, a toxin may be produced and induce peribronchiolar lymphocytic infiltration (Livingston et al., 1972; Underdahl et al., 1980), changes in lectin affinity of bronchiolar epithelium (Ackermann et al., 1991) or histochemical alterations of mucus secreting cells (DeBey et al., in press) in infected swine.

Further evidence for the necessity of colonization is that noncytotoxic strains of M. hyopneumoniae exhibited little or no evidence of colonization by immunofluorescence or TEM, while cytotoxic strains exhibited strong immunofluorescence and clusters of organisms were associated with cilia on TEM. Direct interaction with some component of the host cell membrane may be the mechanism by which cytotoxicity of M. hyopneumoniae is induced in this model.

Colonization of tracheal ring epithelium by M. hyopneumoniae in the presence of CSS was detected by immunofluorescence and TEM 5 days after inoculation. The high numbers of organisms inoculated into and proliferating within the organ culture system may have overwhelmed antibody capable of blocking attachment to ciliated cells. Another explanation for colonization in the presence of CSS is that antibodies protecting against ciliated cell damage may not block attachment. Evidence for this hypothesis has been indirectly corroborated by protection conferred with commercial mycoplasma vaccines currently in use. Lesion severity was reduced in vaccinated pigs, but colonization of airways by M. hyopneumoniae was not averted by vaccination (Dayalu and Ross, 1990; Peterson *et al.*, 1990).

A critical number of organisms is apparently necessary for initial colonization of tracheal organ cultures by M. hyopneumoniae. Ciliary loss was delayed when insufficient

numbers of viable mycoplasma were present in the inoculum. Low numbers of M. hyopneumoniae which managed to pass through a 0.1 um membrane separating M. hyopneumoniae from tracheal rings failed to colonize or induce loss of cilia.

In vitro passage of M. hyopneumoniae significantly decreased the cytotoxic potential of the organism for newborn piglet tracheal organ cultures. Apparently, broth passage of M. hyopneumoniae causes reduction of virulence, while tissue passage enhances virulence. The environment provided by Friis medium and standard incubation conditions may cause down regulation of a gene coding for a protein needed to induce cytotoxic potential. Alternatively, spontaneous deletions such as those described for Escherichia coli (Hacker et al., 1990), or antigenic variation may occur during growth leading to loss of virulence. Variation in the V-1 surface antigen of M. pulmonis occurs in vitro and in vivo (Talkington et al., 1989). Presence of a similar protein in M. hyopneumoniae has not been reported. Loss of a plasmid leads to loss of virulence of Yersinia pseudotuberculosis, because the plasmid contains the gene for an essential protein-tyrosine phosphatase (Bliska et al., 1991). However, no plasmids associated with virulence have been identified for M. hyopneumoniae.

Trypsin- and neuraminidase-sensitive structures present on the surface of human larynx (HEp-2) epithelial cells

induce production of new proteins by Salmonella choleraesuis and S. typhimurium (Finlay et al., 1989a, 1989b).

Apparently, this mechanism is not operative in the M. hyopneumoniae/tracheal epithelium model. Cytotoxicity of M. hyopneumoniae was not enhanced by the presence of lung preparations in the growth medium. Noncytotoxic strain 232C was not induced to become virulent in the presence of lung homogenate. The concentration of heated Mh, NLH or a factor in the lung preparations may have been too low to induce signal transduction. Another possibility is that the factors involved in in vivo enhancement of virulence may be short-lived and unable to withstand conditions used for inactivation of mycoplasmas or collection and preparation of the lung homogenates.

These results may indicate that loss of virulence during in vitro passage is due to culture conditions, rather than loss of essential materials from the lung environment. Growth conditions are known to alter the virulence of Salmonella species. Salmonella cells in the stationary phase of growth were unable to invade Madin-Darby kidney cells in vitro, and oxygen-limited conditions during growth enhanced adherence and invasiveness of the bacteria (Lee and Falkow, 1990). Environmental factors including low temperature, $MgSO_4$, and nicotinic acid modulate expression of proteins associated with virulence of Bordetella pertussis through

signal transduction of gene products of the bvg locus (Arico et al., 1989). Modification of growth medium and/or incubation conditions may be useful for maintenance of virulence of M. hyopneumoniae in vitro.

Calmodulin and dehydrogenase enzyme content of tracheal explant epithelium was not altered by the presence of M. hyopneumoniae, and was not indicative of epithelial damage in this model. In vitro healing, observed with light microscopy and TEM as a single layer of flat epithelial cells, was evident in tissue sections of damaged porcine explant epithelia 5 days after inoculation with M. hyopneumoniae. Epithelial cell damage by M. hyopneumoniae may not be reflected by calmodulin or dehydrogenase enzyme levels because enzyme activity of metabolically active cells participating in the healing response is measured along with that of dying cells.

The present experiment demonstrates that attachment of M. hyopneumoniae to ciliated epithelium is necessary to induce ciliostasis and loss of cilia in this model. Interaction of M. hyopneumoniae with porcine tracheal rings may imitate the relationship of pathogen to host cell in the infected pig. This system may provide a means for assessment of virulence mechanisms in M. hyopneumoniae. Evidence that attachment is necessary for induction of ciliary damage occurring during M. hyopneumoniae infection in vitro further

demonstrates the importance of identifying the immunogens associated with attachment of M. hyopneumoniae in vivo for production of vaccines that prevent infection, as well as pneumonic lesions, in the pig.

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Figure 1. Loss of cilia from porcine tracheal ring epithelium after inoculation with in vivo grown Mycoplasma hyopneumoniae (Mh) from the lung of a pig infected with M. hyopneumoniae, irradiated Mh (Ir Mh) or lung homogenate from a noninfected pig (NIP). On the day of inoculation, tracheal rings inoculated with Mh possessed more epithelium with cilia than rings inoculated with Ir Mh or NIP (a, $P < 0.04$). There was no difference in the percent of epithelium with cilia 1, 2 and 3 days after inoculation. Four and 5 days after inoculation, tracheal rings inoculated with Mh exhibited marked loss of cilia when compared to Ir Mh and NIP (b, $P < 0.001$). Results are means \pm standard error of the means for 3 experiments.

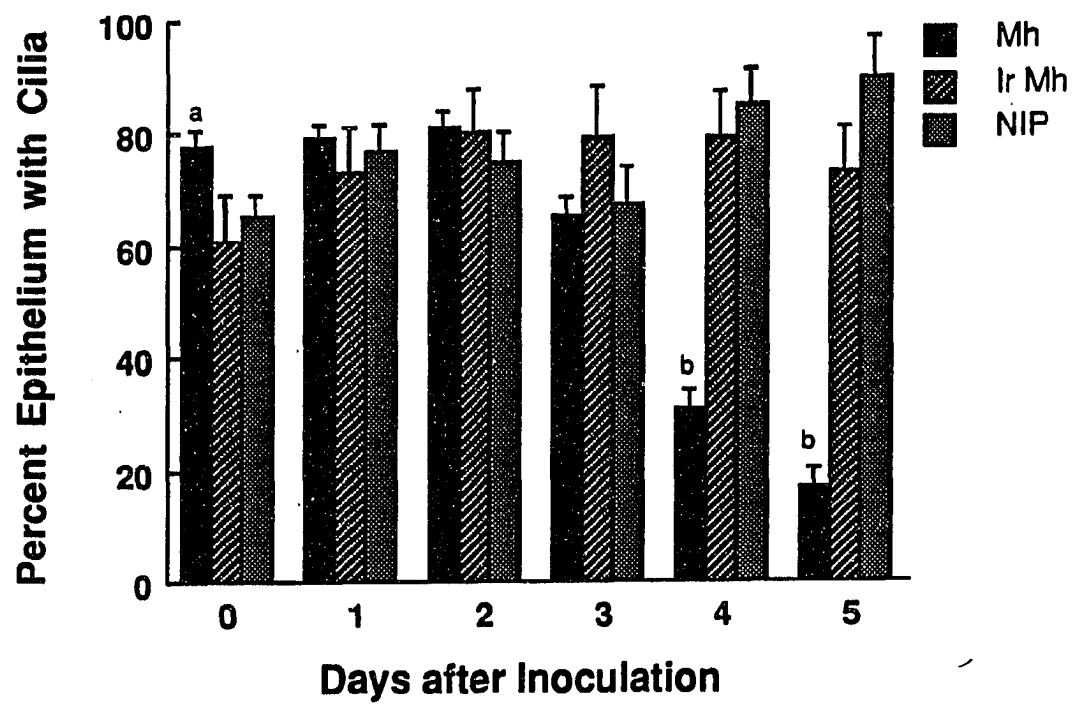


Figure 2. Loss of cilia from porcine tracheal ring epithelium after inoculation with Mycoplasma hyopneumoniae (passage 2; MHP) with and without irradiated lung homogenate from a pig infected with M. hyopneumoniae (Ir Mh). On the day of and the day after inoculation, tracheal rings inoculated with MHP possessed more epithelium with cilia than rings inoculated with Ir Mh only (a, $P < 0.005$). Two days after inoculation, tracheal rings inoculated with MHP + Ir Mh exhibited significantly greater loss of cilia than rings inoculated with MHP or Ir Mh (b, $P < 0.05$). Loss of cilia from rings inoculated with MHP or MHP + Ir Mh was not significantly different for the remainder of the experiment (c, significantly less than Ir Mh, $P < 0.04$). Results are means \pm standard error of the means for 1 experiment ($N = 14$ for each data point). Ciliary loss after inoculation with MHP or MHP + Ir Mh, and inability of Ir Mh to induce epithelial damage in tracheal rings, were independently confirmed in 3 or more experiments.

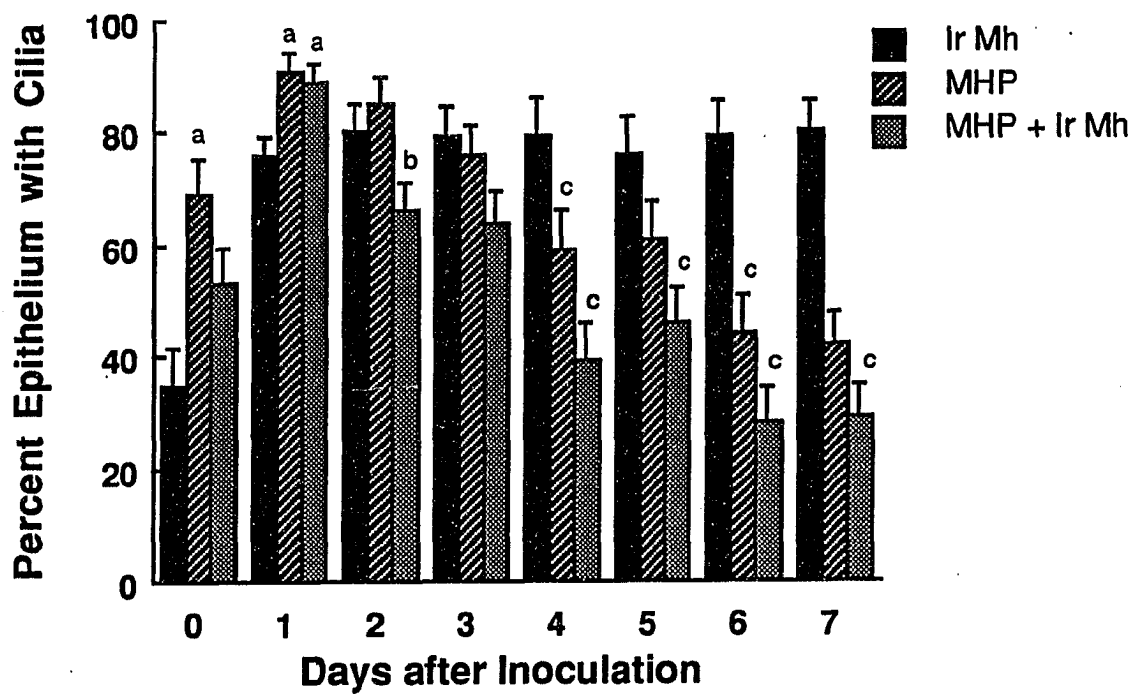


Figure 3. Colonization of tracheal ring epithelium by Mycoplasma hyopneumoniae (M) 5 days after inoculation. Mycoplasma cells are closely associated with cilia (C). Bar = 0.83 μ m.



Figure 4. - Days after inoculation of porcine tracheal rings until onset of significant ciliary loss (*, $P < 0.05$ when compared to other dilutions). Tracheal rings were inoculated with tenfold dilutions of Mycoplasma hyopneumoniae and the percent of epithelium retaining cilia was recorded daily. Results are means +/- standard error of the means for 3 experiments.

Onset of Ciliary Loss

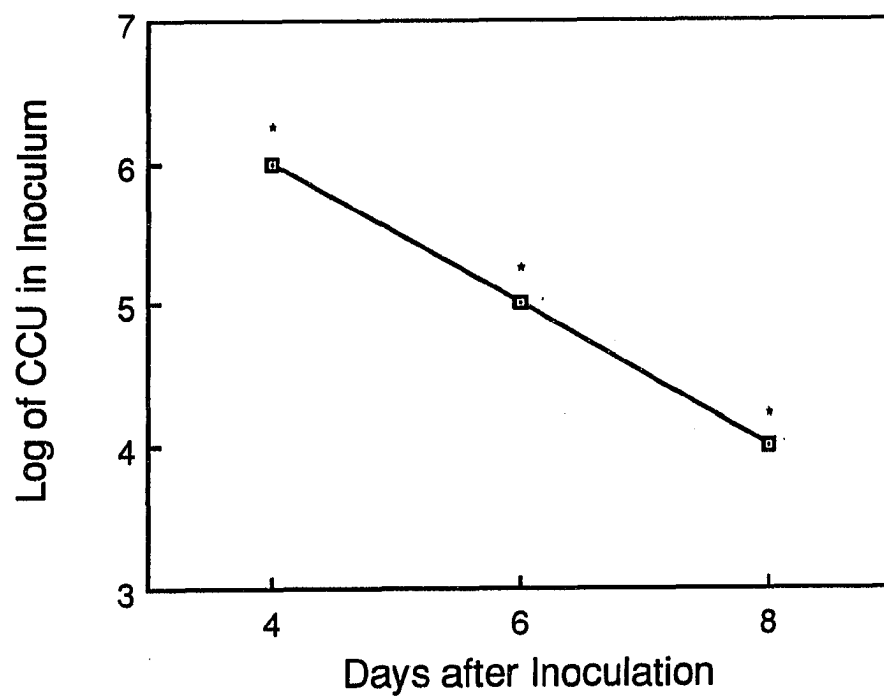


Figure 5. Loss of cilia from tracheal ring epithelium 2 and 5 days after inoculation with Mycoplasma hyopneumoniae passaged in vitro. There was no difference in values for M. hyopneumoniae passages or the control 2 days after inoculation. Five days after inoculation, tracheal rings inoculated with sterile mycoplasma medium retained significantly more ciliated epithelium than all passages (a, $P < 0.009$). Passage 2 induced significantly more ciliary loss than all other passages, except passage 4 (b, $P < 0.02$). Passage 20 was significantly less cytotoxic than passages 2, 4 and 10 (c, $P < 0.05$). Results are means \pm standard error of the means for one experiment which included all passages of M. hyopneumoniae (N = 12 to 16 tracheal rings for each data point). Loss of cytotoxic potential with in vitro passage was confirmed using passages 2, 10 and 20 in another experiment.

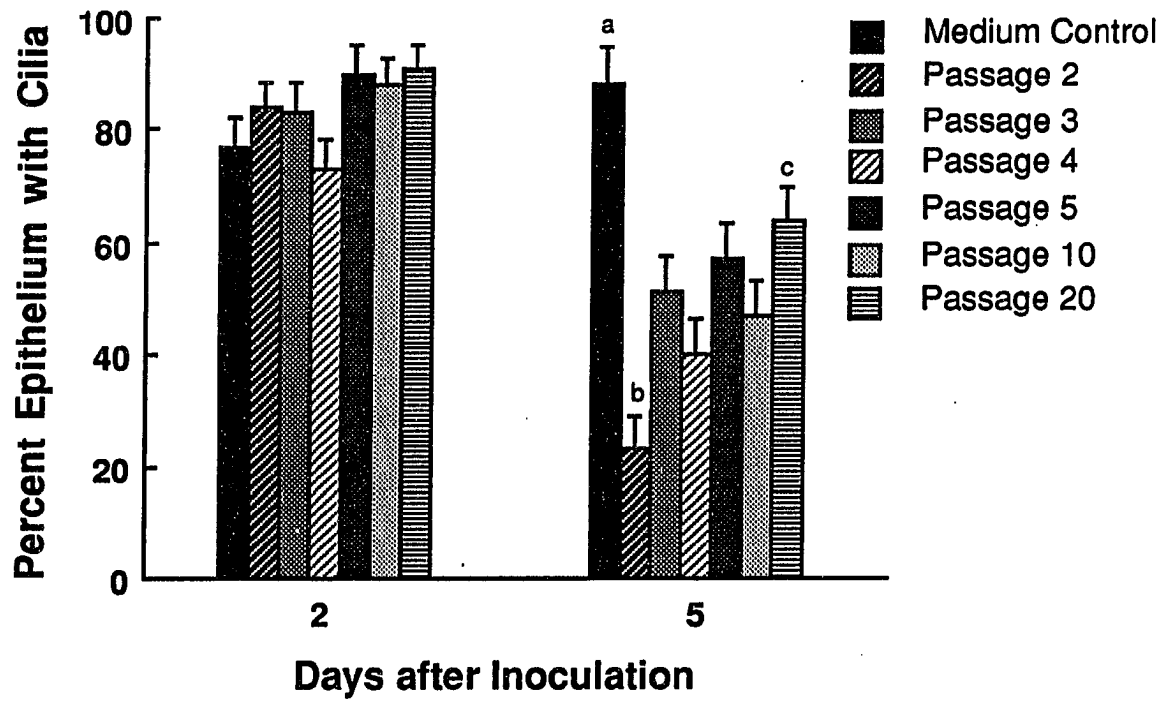


Figure 6. Loss of cilia from porcine tracheal ring epithelium after inoculation with Mycoplasma hyopneumoniae (Mh) with or without normal swine serum (NSS) or convalescent swine serum (CSS). Three, 4 and 5 days after inoculation, tracheal rings inoculated with Mh + CSS containing antibody specific for Mh possessed more epithelium with cilia than rings inoculated with Mh or Mh + NSS ($P < 0.02$). Results are means +/- standard error of the means for 2 experiments.

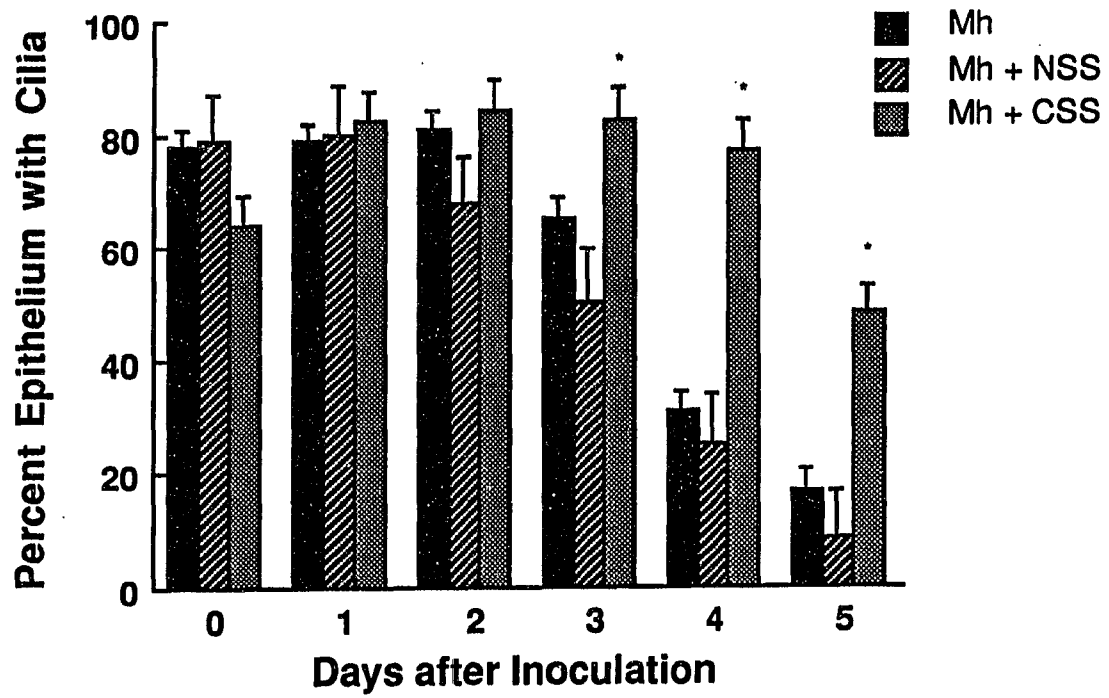
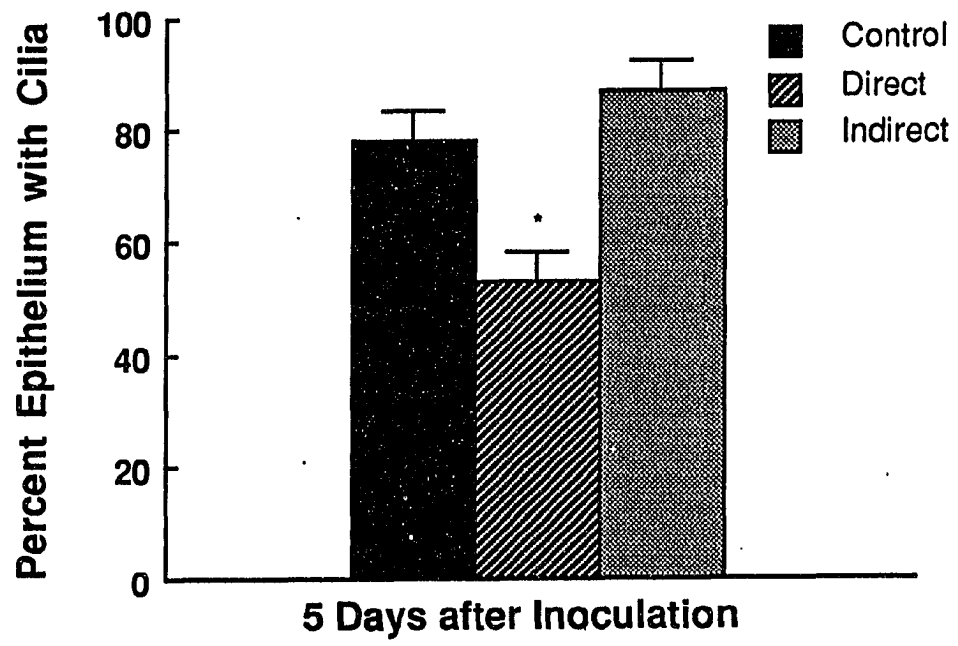


Figure 7. Loss of cilia after direct inoculation of porcine tracheal rings with Mycoplasma hyopneumoniae (Direct), but not after indirect exposure to mycoplasmas through a membrane with 0.1 um pore size (Indirect). Each tracheal ring was placed on top of the membrane of a well insert. Mycoplasma hyopneumoniae was inoculated above and below (Direct) or only below (Indirect) the membrane. Loss of cilia from tracheal rings separated from M. hyopneumoniae by the membrane and from rings inoculated with sterile mycoplasma medium (Control) was not significantly different. Tracheal rings directly inoculated with M. hyopneumoniae exhibited significant loss of cilia (*, $P < 0.002$). Results are means +/- standard error of the means for 2 experiments.



SECTION III: ALTERATION OF NEUTROPHIL INTRACELLULAR CALCIUM
CONCENTRATION BY MYCOPLASMA HYOPNEUMONIAE

Alteration of Neutrophil Intracellular Calcium
Concentration by Mycoplasma hyopneumoniae

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ABSTRACT

Neutrophils were isolated from the peripheral blood of pigs free of infection with Mycoplasma hyopneumoniae, and loaded with fluorescent indicator (Fura-2) for detection of cytosolic free calcium. Kinetics of intracellular calcium flux, measured with a photon-counting spectrofluorometer, were examined after incubation with or without M. hyopneumoniae strains pathogenic or nonpathogenic for pigs by intratracheal inoculation. Basal intracellular calcium concentration was not altered by M. hyopneumoniae. After zymosan stimulation, intracellular calcium levels were significantly increased in neutrophils incubated with M. hyopneumoniae. Mycoplasma hyopneumoniae enhanced the porcine neutrophil response to opsonized zymosan, suggesting that the organism had modulated calcium dependent response elements.

INTRODUCTION

Infection of swine by Mycoplasma hyopneumoniae is apparently widespread throughout the world (Gois et al., 1980; Yamamoto and Ogata, 1982; Ross, 1986), and pneumonia induced by the organism is often complicated by secondary bacteria (Gois et al., 1975; Ross, 1986). Histologically, large numbers of neutrophils are commonly observed in bronchiolar lumina (Livingston et al., 1972; Ross, 1986). The effect of M. hyopneumoniae on neutrophil function, or alternatively, the role of neutrophils in mediating lesion development or resolution is not known.

Changes in intracellular calcium concentrations ($[Ca]_i$) modulate cellular activity. Increases in $[Ca]_i$ usually indicate activation of neutrophils (Rasmussen et al., 1985).

Zymosan opsonized with porcine serum stimulates porcine neutrophils (Goff et al., 1991), probably via the C3b receptor which apparently activates a G protein. Activated G protein may modulate efflux of intracellular calcium from endoplasmic reticulum (Rink, 1987) or influx of extracellular calcium through calcium channels to the cytosol (reviewed in Sternweis and Pang, 1990). In this study we demonstrated enhanced concentrations of cytosolic calcium in porcine neutrophils incubated with M. hyopneumoniae and stimulated with opsonized zymosan.

MATERIALS AND METHODS

Pigs. Fifteen Yorkshire crossbred pigs 2-4 months old were obtained from a barrier maintained, closed herd which is free of infection with known swine respiratory pathogens. The herd, located at the animal resource station at Iowa State University, was originally established from Caesarean born, isolation reared swine. For this study pigs were housed in isolation rooms alone or in groups of 2 to 4 animals, and fed a 16% protein swine grower ration without antibacterials. Sera collected from the pigs did not contain antibody to M. hyopneumoniae as determined by the ELISA test (Bereiter et al., 1990).

Neutrophil isolation. Neutrophils were isolated as previously described (Goff et al., 1991), with modifications. Briefly, 30 ml cold (4 C) citrated peripheral blood was mixed with 100 ml cold phosphate buffered deionized water (PBW; 0.0132 M, pH 7.2) for 90 seconds, then 50 ml cold phosphate buffered (0.0132 M, pH 7.2) 2.7% saline (PBN) for 20 seconds, and centrifuged at 365 x g for 15 minutes. Approximately 150 ml of supernate was removed, cells were resuspended and the lysis step was repeated. After centrifugation, approximately 160-170 ml of supernate was removed, the cells were resuspended in the remaining liquid, the suspension was layered on top of 6 ml cold Ficoll (Histopaque 1077, Cat. No. H8889, Sigma Chemical Co., St. Louis, MO), and centrifuged at

400 x g for 45 minutes. Supernate, mononuclear cells, and approximately 3.0 ml of the Ficoll was removed. If large numbers of red blood cells were apparent, the cell suspension was mixed with 10 ml of cold PBW for 60 sec, then 5 ml of cold PBN for 20 seconds, before washing and counting in cold 0.015 M phosphate-buffered saline solution, pH 7.2 (PBS). If few red blood cells were present, cells were washed in approximately 15 ml PBS before counting. All cells were washed in PBS after counting, and resuspended in PBS to a concentration of 5.0×10^7 cells per ml. This procedure generally yielded cell preparations that were greater than 90% neutrophils.

Mycoplasma hyopneumoniae. Lung homogenate from a pig infected with M. hyopneumoniae strain 232 was diluted 1:10 in Friis mycoplasma medium containing antibacterials (Friis, 1975) and incubated until the logarithmic phase of growth to obtain passage 1. Passage 1 was diluted 1:10 in Friis mycoplasma medium and the culture was incubated to obtain passage 2. Aliquots of passages 1 and 2 were frozen at -70 C. Passage 2 of M. hyopneumoniae strain 232 induced ciliostasis in porcine tracheal organ cultures (DeBey and Ross, 1990) and caused lesions in the lungs of pigs inoculated intratracheally (R.F. Ross, 1990). Another culture of M. hyopneumoniae (strain J, ATCC 25934) was passaged in Friis mycoplasma medium and aliquots of passages

55-58 were frozen at -70 C. Ciliostasis in tracheal organ cultures was not induced by passages 55-63 of this strain (DeBey and Ross, 1989). At passage 60, M. hyopneumoniae strain J was not pathogenic for pigs when inoculated intratracheally (Zielinski and Ross, 1990).

For preparation of fresh cultures, an aliquot of frozen culture was inoculated into Friis mycoplasma medium without antibacterials the day before an experiment, and incubated at 37 C with rotation to obtain passage 59 of strain J and passage 2 of strain 232. In some experiments frozen cultures were thawed and used the day of the experiment.

Fresh or frozen/thawed cultures of M. hyopneumoniae were centrifuged at 10,000 x g for 15 minutes and the cell pellet was resuspended in 50% Friis mycoplasma medium without antibacterials and 50% PBS (incubation medium). The suspension was incubated for 2-4 hours at 37 C with constant rotation. Before addition to neutrophils, the mycoplasmas were centrifuged at 10,000 x g for 15 minutes and resuspended in fresh incubation medium. Immediately prior to inoculation of neutrophils, numbers of organisms in the suspension were estimated by the tube dilution method of determination of color changing units (CCU) for each mycoplasma. One CCU represented the highest dilution of culture changing the color of a tube of Friis medium from red to yellow following incubation for 2-42 days.

Cytoplasmic calcium flux. Kinetics of intracellular calcium flux were measured using an SLM 8000C photon-counting spectrofluorometer (SLM Instruments, Inc., Urbana, IL) as described by Grynkiewicz et al. (1985). This instrument has three photomultiplier tubes in T format optics and a stirred thermostated sample chamber with an injection port for addition of zymosan during the assay. The SLM 8000C is controlled by an IBM PC computer interfaced to a Hewlett-Packard 7470A plotter.

Neutrophils from each pig were loaded with fluorescent indicator by incubating 5×10^7 neutrophils/ml with 10 ug/ml Fura-2 (Calbiochem-Behring Diagnostics, San Diego, CA) in PBS in the dark for 30 min at 37 C while tumbling end over end in polystyrene tubes (loaded cells). Neutrophils suspended in PBS without Fura-2 (5×10^7 cells/ml; blank cells) were incubated while tumbling in the same manner. After incubation, loaded and blank cells were washed in PBS and resuspended to 5.0×10^7 neutrophils/ml in incubation medium (50% Friis mycoplasma medium/50% PBS) with or without M. hyopneumoniae. Suspensions were incubated for 2 hours in the dark with constant rotation, and mixed more vigorously to resuspend neutrophils once during incubation. After incubation, cells were pelleted at $180 \times g$ for 15 minutes and resuspended to 5.0×10^7 neutrophils/ml in PBS. Then 5.0×10^6 neutrophils were added to 2.5 ml prewarmed (39 C)

Hank's balanced salt solution (HBSS) without phenol red (Gibco Lab., Grand Island, NY) in a cuvette with a stir bar. Correction for autofluorescence of neutrophils or mycoplasmas was performed by calibration of the system with appropriate non-Fura-2 loaded cells with or without mycoplasma before testing Fura-2 loaded cells. Fluorescence ratios were recorded every 2 seconds for 98 seconds to obtain original spectra. Opsonized zymosan was added to the Fura-2 loaded cell suspension 10 sec after initiation of the assay. The original spectra obtained during the experiment were mathematically adjusted to a baseline value of 1 fluorescence unit 5 sec after initiation of the assay to compare $[Ca]_i$ of neutrophils after zymosan stimulation. Original and adjusted fluorescence ratios were plotted over a 98 sec time period.

Statistical methods. Analysis of variance of the fluorescence ratios of original spectra at 6 seconds (4 seconds before zymosan stimulation) was used for detection of differences in basal $[Ca]_i$ of neutrophils. Least significant difference ($p < 0.05$) was calculated to compare means obtained for pathogenic (232) and nonpathogenic (J) M. hyopneumoniae and uninoculated controls. Data were pooled for analysis.

A quadratic equation was calculated from all adjusted data acquired from 20-98 seconds (all data acquired 10-88 seconds after zymosan stimulation) to describe each curve by

regression with autocorrelated errors. From the quadratic equation 3 parameters were calculated: intercept, linear coefficient, and the quadratic coefficient to estimate slope (slant) and curvature (shape) of each curve. Analysis of variance was used for detection of differences in curve parameters, and therefore $[Ca]_i$, after zymosan stimulation. Least significant difference ($p < 0.05$) was calculated to compare curve parameters of pathogenic and nonpathogenic M. hyopneumoniae and uninoculated controls. Each experiment was analyzed separately.

RESULTS

Mycoplasma hyopneumoniae. Neutrophils serving as controls were suspended in medium without mycoplasmas. Neutrophils inoculated with mycoplasmas were suspended in medium containing 10^7 to 10^{10} CCU of M. hyopneumoniae. For each experiment, concentration of M. hyopneumoniae 232 was within 1 ten-fold dilution of the J strain.

Cytoplasmic calcium flux. Basal $[Ca]_i$ of porcine neutrophils was not altered by incubation with M. hyopneumoniae. Using the linear coefficient, which measured the way the curve changed over time, kinetics of $[Ca]_i$ was significantly different for neutrophils with and without mycoplasma. Increases in $[Ca]_i$ were significantly greater in neutrophil suspensions inoculated with M. hyopneumoniae than in uninoculated controls after zymosan stimulation ($p = 0.0062$). After zymosan stimulation free $[Ca]_i$ was significantly higher ($p = 0.0192$) in neutrophils incubated with pathogenic M. hyopneumoniae than in neutrophils incubated with the nonpathogenic J strain when quadratic coefficients displayed little variability between animals in an experiment. See Figure 1 for spectra from a representative sample.

Discussion

Neutrophils exposed to M. hyopneumoniae exhibited significantly greater $[Ca]_i$ in response to stimulation with opsonized zymosan than uninoculated control neutrophils. Rapidly increasing $[Ca]_i$ may be due to increased flux of calcium across the cell membrane from extracellular sources, or release of calcium from intracellular stores (Rink, 1987).

Mycoplasma hyopneumoniae modulated neutrophil response to the opsonized zymosan stimulus, without altering basal $[Ca]_i$. Results suggest that M. hyopneumoniae up-regulated or impaired the elements regulating response of neutrophils to zymosan stimulation, and imply that the neutrophils were "primed" to exhibit exaggerated intracellular calcium flux in response to stimulation of the C3b receptor. The activity of one or more calcium-dependent response elements may be altered without changing $[Ca]_i$ (Rasmussen et al., 1985). Priming of human neutrophils by diacylglycerol (Liang et al., 1990) and nucleotides (Walker et al., 1991) for enhanced $[Ca]_i$ flux in response to stimulants has been described.

Calcium channels are inhibited by fatty acids (McPhail et al., 1984; Sekiguchi et al., 1987; Hwang et al., 1990; Ordway et al., 1991). Fatty acids maximally inhibitory of calcium channel activity, myristic, oleic and linoleic acid (reviewed in Ordway et al., 1991) compose approximately 47% of the total membrane lipid of M. hyopneumoniae (Hwang et

al., 1986). However, the mechanism for release of free fatty acids from mycoplasma membranes and subsequent transfer to host cell membrane proteins is not clear.

The role of neutrophils in pathogenesis of M. hyopneumoniae disease is not known. Neutrophil products may be involved in acute lung injury. Accumulation of neutrophils in airways (Livingston et al., 1972; Ross, 1986) coincides with epithelial cell injury (Livingston et al., 1972) and apparent immunosuppression (Adegboye, 1978a and b) observed during acute and mid-stage disease. Intracellular calcium fluxes, such as those observed by us, are associated with activation of cells (Rasmussen et al., 1985). Killing of murine type II pneumocytes by oxygen metabolites, typically produced by activated neutrophils, was dose dependent and cellular function was impaired by concentrations equivalent to the amount produced by in vivo neutrophil:type II pneumocyte ratios of 3:1 to 6:1 (Crim and Simon, 1988).

Killing of type II pneumocytes by oxygen metabolites was preceded by altered function of these cells (Crim and Simon, 1988). Crim and Simon (1988) suggested that such alterations might reduce the ability of these pneumocytes to produce surfactant. Evidence of reduced surfactant system function in the lungs of pigs infected with M. hyopneumoniae was reported by Wichert and Wilke (1976). Surfactant content was

not quantitated. However, the authors stated that impairment of surfactant system function by edema in affected lungs was more likely than reduced production of surfactant.

The results of our study suggest that M. hyopneumoniae alters neutrophil intracellular calcium flux in response to an opsonized zymosan stimulus. The mechanisms for increased $[Ca]_i$ may include enhanced efflux of calcium from intracellular stores and/or enhanced influx of calcium from extracellular sources, and suggests modulation of calcium dependent response elements by M. hyopneumoniae. The enhanced $[Ca]_i$ response may indicate that neutrophils hypersensitive to stimuli cause increased neutrophil-induced lung damage when mycoplasma are present. Determination of the mechanism inducing increased free $[Ca]_i$ in neutrophils exposed to M. hyopneumoniae may reveal mechanisms of pathogenicity of this organism in vivo.

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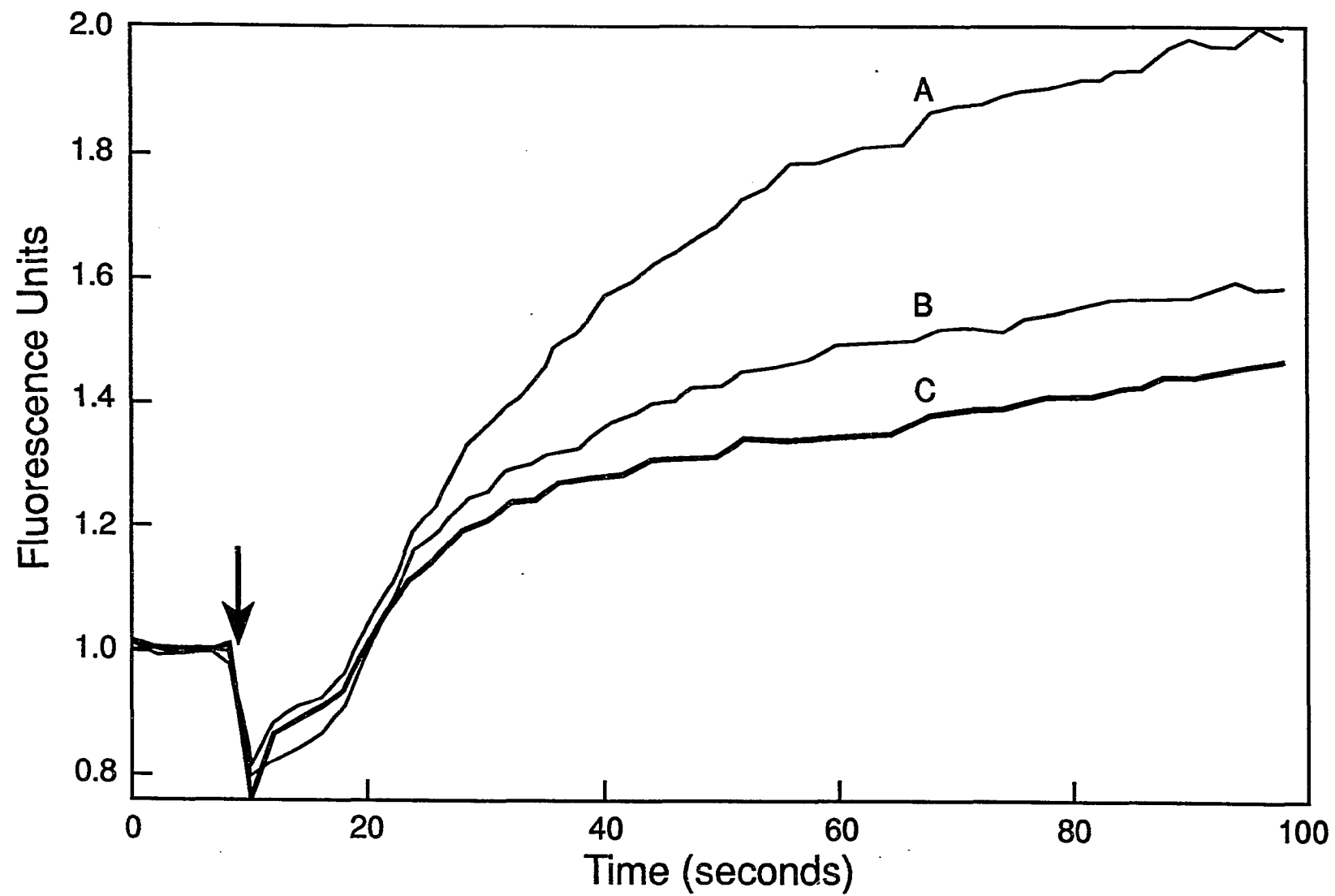
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Figure 1. Effect of Mycoplasma hyopneumoniae on intracellular calcium flux in porcine neutrophils stimulated with porcine opsonized zymosan (arrow). Lines are tracings of fluorescence ratios exhibited by neutrophils without (C) or with pathogenic (A) or nonpathogenic (B) strains of M. hyopneumoniae. Neutrophils loaded with a fluorescent indicator (Fura-2) were incubated for 2 hours at 37 C with or without M. hyopneumoniae. Intracellular calcium flux was measured with a dual wavelength photon-counting spectrofluorometer. Fluorescence ratios were calculated over a total of 98 sec and mathematically adjusted to a baseline value of 1 fluorescence unit at 5 sec. Neutrophils incubated with M. hyopneumoniae consistently exhibited higher levels of intracellular calcium after zymosan stimulation ($P < 0.02$). Representative fluorescence ratios of neutrophils from a single pig are presented.



GENERAL DISCUSSION

Attachment of M. hyopneumoniae to cilia of porcine tracheal ring epithelium was necessary to induce ciliostasis and loss of cilia. A high passage strain of M. hyopneumoniae (232, cloned) failed to attach to cilia or induce ciliated cell damage in porcine tracheal ring epithelium. However, high and low passages of M. hyopneumoniae successfully attached to isolated porcine ciliated cells (Zielinski, 1991). The reason for this discrepancy in results is unclear. Ciliary activity of porcine tracheal ring epithelium may be an important factor preventing attachment of high passage strains of M. hyopneumoniae. Colonization of actively beating cilia may entail a different process or an extra step for successful completion.

Metabolically active or growing cells were required to induce epithelial damage, because irradiated organisms did not induce significant changes in tracheal ring epithelial cells when compared to controls. Perhaps the organism must multiply to a critical number or density on the cilia to provide enough of the cytotoxic component.

Cytotoxicity of M. hyopneumoniae for ciliated cells of porcine tracheal rings was decreased by in vitro passage. Cultivation in vitro evidently causes a change in regulation of expression of a protein essential for colonization of tracheal ring epithelium. This protein could be a membrane

component, or an enzyme necessary for synthesis or transport of a "colonization factor" to the membrane surface. The M. hyopneumoniae toxic protein previously described by Geary and Walczak (1983, 1985) is probably not the putative "colonization factor", because in vitro passage did not affect expression of that toxin. However, the toxin they described may be important for induction of ciliostasis and loss of cilia once attachment and colonization have occurred.

The enhancement of $[Ca]_i$ observed in porcine neutrophils exposed to M. hyopneumoniae might occur via modulation of protein kinase C, calcium channel proteins or other calcium-dependent response elements, such as calmodulin. Fatty acids capable of activating protein kinase C or inhibiting calcium channels comprise a significant portion of the membrane lipids of M. hyopneumoniae. Perhaps fatty acids contained in lipids of the mycoplasma membrane are important for cytotoxicity. However, the mechanism for cytotoxicity to ciliated cells by fatty acids of mycoplasma membrane lipids is not clear. Intracellular calmodulin level in tracheal ring epithelium inoculated with M. hyopneumoniae was not significantly different from controls. However, sample preparation for this assay would have destroyed any heat-sensitive protein/factor affecting calmodulin function. Therefore, alteration of calmodulin function by M. hyopneumoniae was not assessed in our experiment.

The increased exudate present in airways of M. hyopneumoniae-infected pigs at necropsy, as well as a previous report of decreased surfactant function of mucus from infected pigs (Wichert and Wilke, 1976), led us to investigate the histochemical characteristics of mucus within submucosal glands and goblet cells of infected and noninfected pigs. The decreased area of epithelium occupied by goblet cells of infected pigs may have indicated that the contents were rapidly released to the airway lumen, rather than stored in the cell, after synthesis. Increased sulphation of mucus may indicate local irritation and hypersecretion in response to hydrogen peroxide or other molecules produced by M. hyopneumoniae organisms intimately associated with the epithelium. Goblet cells respond to local stimuli, eg., irritation, while submucosal glands are probably neurologically regulated (Reid et al., 1983).

Microscopic lesions consisting of lymphocytic infiltration, altered histochemical staining of mucous glycoprotein, and hyperplasia and ciliary exfoliation of epithelial cells provide indirect evidence that M. hyopneumoniae can induce changes in multiple cell types. Data presented in this dissertation demonstrated that M. hyopneumoniae can directly affect the function of ciliated epithelial cells and neutrophils. The ability to directly induce changes in more than one cell type may indicate that

M. hyopneumoniae acts on a membrane protein that is common to these cell types.

The nature of the mycoplasma binding site and the host cell receptor may aid in identification of the mechanisms by which M. hyopneumoniae induces epithelial cell damage. However, the binding site and the toxic component of M. hyopneumoniae are probably different molecules. Further research should be done to elucidate the molecule or molecules responsible for attachment of M. hyopneumoniae in vitro, and determine if they are also important in vivo. Identification of the host receptor molecule(s) may give insight to mechanisms of pathogenicity used by this organism to cause disease. Development of a medium for growth of M. hyopneumoniae which does not cause down-regulation of the cytotoxic potential of the organism for tracheal ring epithelium may assist in identification of colonization and toxigenic factors necessary for induction of ciliostasis and loss of cilia.

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APPENDIX A: EFFECT OF CYTOTOXIC MYCOPLASMA HYOPNEUMONIAE ON
TRACHEAL ORGAN CULTURES

Cytotoxic effects of M. hyopneumoniae were evaluated in porcine tracheal rings. This appendix contains data not shown in the manuscript entitled "Ciliostasis and Loss of Cilia Induced by Mycoplasma hyopneumoniae in Porcine Tracheal Organ Cultures", pages 81-127 of this dissertation. The manuscript contains more detailed methods concerning mycoplasma, tracheal ring and lung extract preparation.

MATERIALS AND METHODS

Lung homogenate and M. hyopneumoniae. Lung homogenate (LH) containing M. hyopneumoniae was obtained from a pig infected with M. hyopneumoniae strain 232, a derivative of strain 11. The homogenate contained 10% (w/w) lung tissue in Friis mycoplasma medium without antibacterials, and served as a source of in vivo grown M. hyopneumoniae (Mh). The Mh was free of mycoplasmas other than M. hyopneumoniae or other bacteria, and negative for viruses by transmission electron microscopy (TEM) and immune TEM. A portion of Mh was subjected to 180,000 rad (Ir Mh). Supernate (Cent-Irrad) was prepared by centrifugation of Ir Mh at 740 x g for 15 minutes. Supernate of Mh was prepared by heating at 100 C for 2 minutes and centrifugation at 740 x g for 15 minutes (heated Mh). Filtrate (Fil-10) and retentate (Ret-10) were prepared by passing heated Mh through a filter retaining

molecules greater than approximately 10,000 molecular weight. Lung homogenate collected from a normal pig, free of infection with M. hyopneumoniae, was prepared as a 10% (w/w) suspension in Friis mycoplasma broth. Aliquots of lung preparations were stored at -70 C until used.

The Mh was diluted tenfold in Friis mycoplasma broth, incubated, passaged in Friis broth and stored at -70 C to provide second (MHP), third, fourth, fifth, tenth and twentieth passage M. hyopneumoniae. Strain 232, used for inoculation of a pig to produce Mh, was cloned (clone 2A3; 232C), grown to passage 40 in Friis broth and frozen at -70 C. At passage 36 232C induced pneumonic lesions in pigs inoculated intratracheally (See manuscript, pages 81-127). For inoculation to tracheal rings, frozen cultures were thawed and diluted in Friis mycoplasma broth without thallium acetate.

Tracheal Rings. Crossbred Hampshire and Yorkshire piglets were obtained from sows from commercial swineherds. Each piglet was deeply anesthetized and the trachea aseptically removed at the level of the right bronchus. The excised trachea was cut into 20-25 rings. Each ring was randomly assigned to a well in a 24 well tissue culture plate. Rings were incubated with or without M. hyopneumoniae in Friis medium without thallium acetate at 35.5 C in 0.5% CO₂.

Experimental design. Appropriate control or mycoplasma preparation in 0.3 ml Friis medium was randomly assigned to each well of a 24 well tissue culture plate. Spent medium was removed daily and 0.3 ml fresh Friis medium added to each well until the ring was removed or the experiment was terminated. The medium from each well containing a ring was cultured for M. hyopneumoniae and other bacteria whenever a ring was removed.

Direct evaluation of damage to ciliated epithelium.

Cytotoxicity of M. hyopneumoniae for tracheal epithelium, manifest as loss of cilia from epithelial cells and ciliostasis, was evaluated using an inverted microscope (100X) with slight modification of the methods described by Gabridge and Polisky (1976). Loss of cilia was determined after estimating the percent of epithelium retaining ciliated cells (0-100%). Ciliostasis, evaluated by observing the vigor of ciliary beating, was scored on a scale from 0 (no movement) to 3 (vigorous beating).

Evaluation of dehydrogenase enzyme levels in porcine tracheal rings. Reduction of 2,3,5-triphenyl tetrazolium chloride (TTC) by dehydrogenase enzymes of metabolically active cells of tracheal rings was measured. Rings with and without M. hyopneumoniae were evaluated. After incubation with TTC, the ring was placed in a vial containing 1.0 ml methanol for 30 minutes to extract formazan dye resulting from reduction of

TTC. Triplicate 200 μ l aliquots of methanol from each vial were assigned to a 96 well plate and the optical density (OD) was determined using an ELISA plate reader with a 490 nm filter. Dry weight of each tracheal ring was determined and OD/mg dry weight was calculated.

Evaluation of calmodulin levels in tracheal ring epithelium.

Two or five days after inoculation, epithelium from each tracheal ring was dissected from the cartilage, weighed and homogenized. Calmodulin and protein content of heated (100C) tissue homogenate were measured after centrifugation at 740 and 105,000 \times g to assess total or unbound intracellular calmodulin, respectively. Volume of spent medium removed daily from each well was recorded and pooled by well for measurement of extracellular calmodulin content. Pooled medium from each well was cultured for *M. hyopneumoniae* and bacteria, heated (100C), centrifuged (105,000 \times g) and evaluated for calmodulin and protein content.

Calmodulin levels of triplicate samples were assessed indirectly by measuring phosphate release from AMP in a calmodulin activated enzyme cascade. Cellular calmodulin content was calculated with reference to protein content (BCA Protein Assay, Pierce, Rockford, IL) and explant tissue weight. Extracellular calmodulin content was calculated with reference to protein content and total volume of medium.

Electron Microscopy. Selected tracheal rings were fixed at 4 C in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (CB), pH 7.2, postfixed in 1% osmium tetroxide in CB for 2 hours at room temperature, washed with distilled water, dehydrated in a graded series of acetone of 50, 75, 95 and 100% and embedded in Epon 812 resin (Electron Microscopy Sciences, Fort Washington, PA). Thick sections were cut at 1-2 μ m with glass knives and stained with 1% Toluidine blue. Thin sections were cut at 70-90 nm with a diamond knife, stained with 2% uranyl acetate and Reynold's lead citrate and examined in a Hitachi H500 transmission electron microscope at 75 KV.

RESULTS

Data is presented in Figures 1-23 of this appendix.

Figure 1. Ciliostasis induced by in vivo grown Mycoplasma hyopneumoniae (Mh) from the lung of a pig infected with M. hyopneumoniae, irradiated Mh (Ir Mh) or lung homogenate from a noninfected pig (NIP). Four and 5 days after inoculation, tracheal rings inoculated with Mh displayed a marked decrease in vigor of ciliary beating when compared to Ir Mh and NIP (*, $P < 0.001$). Results are means \pm SEM for 3 experiments.

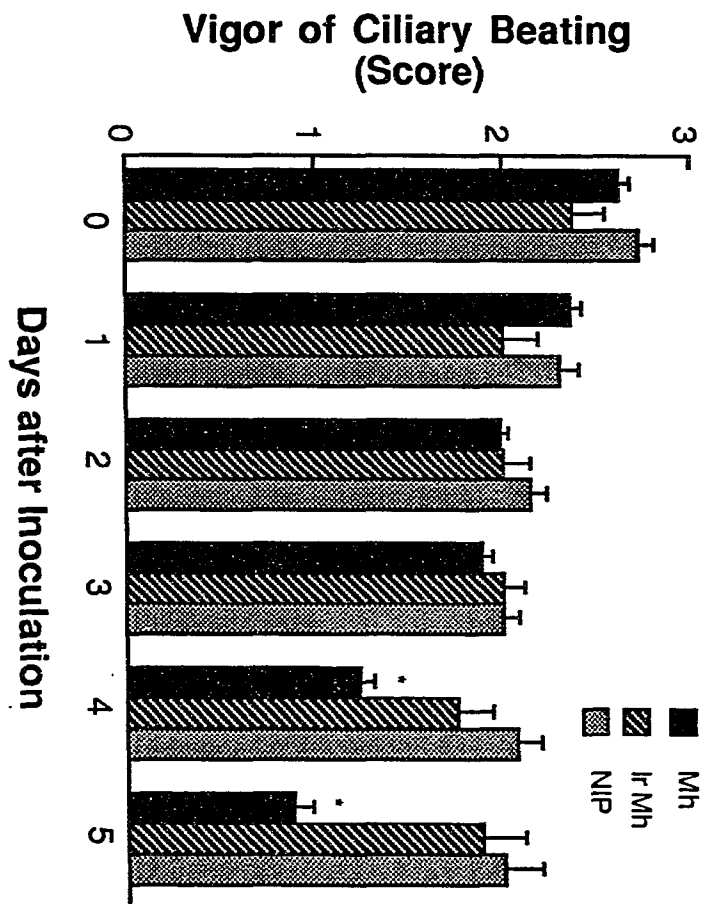


Figure 2. Ciliostasis in porcine tracheal rings induced by Mycoplasma hyopneumoniae (passage 2; MHP) after inoculation with and without irradiated lung homogenate from a pig infected with M. hyopneumoniae (Ir Mh). Three days after inoculation, tracheal rings inoculated with MHP + Ir Mh exhibited significantly more ciliostasis than rings inoculated with MHP or Ir Mh (a, $P < 0.003$). Ciliostasis induced in rings inoculated with MHP or MHP + Ir Mh was not significantly different for the rest of the experiment (b, significantly less than Ir Mh, $P < 0.03$). Results are means \pm SEM for 1 experiment (N = 14 for each data point). Induction of ciliostasis by MHP and MHP + Ir Mh, and inability of Ir Mh to induce ciliostasis in tracheal rings, were independently confirmed in 3 or more experiments.

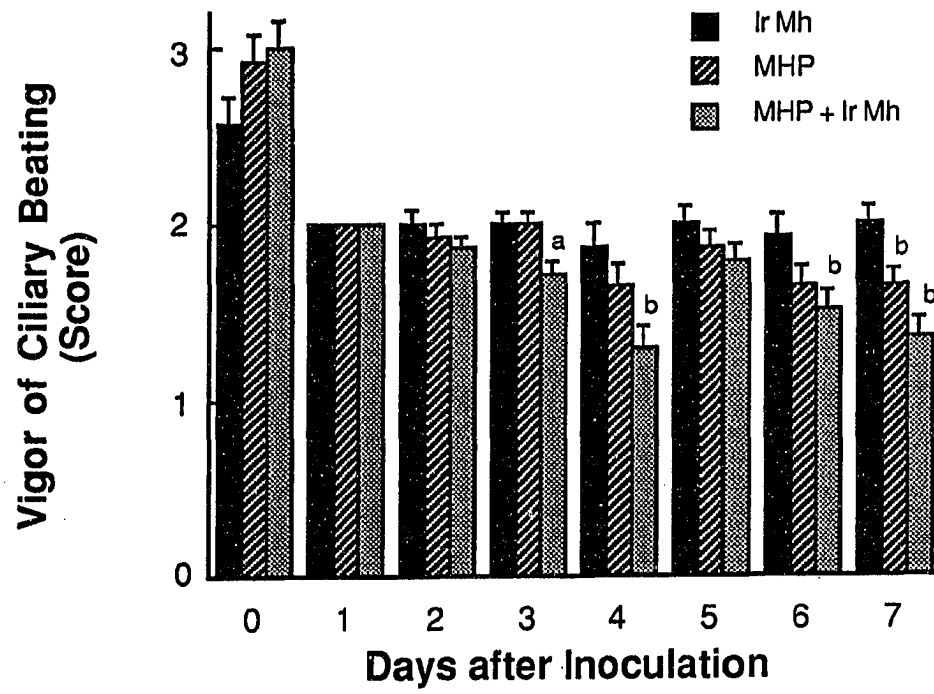


Figure 3. Vigor of ciliary beating on porcine tracheal rings 2 and 5 days after inoculation with Mycoplasma hyopneumoniae passaged in vitro. There was no difference in values 2 days after inoculation. Ability to induce ciliostasis in ciliated epithelium 5 days after inoculation decreased with in vitro passage. Tracheal rings inoculated with sterile mycoplasma medium (control) retained significantly more ciliary activity than all passages, except passages 10 and 20 (a, $P < 0.007$). Passage 2 induced significantly more ciliostasis than all other passages, except passage 4 (b, $P < 0.014$). Passage 20 induced significantly less ciliostasis than all other passages, except passage 10 (c, $P < 0.04$). Results are means \pm SEM for one experiment which included all passages of M. hyopneumoniae (N = 12 to 16 tracheal rings for each data point). Loss of ability to induce ciliostasis with in vitro passage was confirmed for passages 2, 10, and 20 in another experiment.

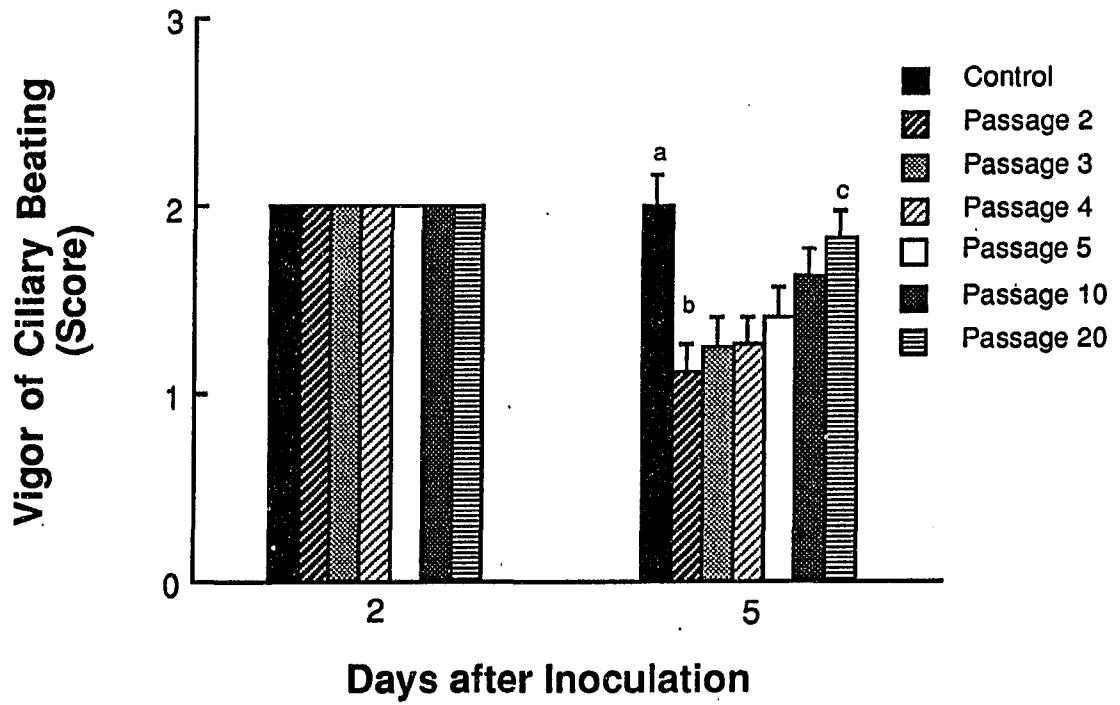


Figure 4. Induction of ciliostasis in porcine tracheal rings by Mycoplasma hyopneumoniae (Mh) with or without normal swine serum (NSS) or convalescent swine serum (CSS). Two and 5 days after inoculation, tracheal rings inoculated with Mh + CSS containing antibody specific for Mh displayed significantly greater vigor of ciliary beating than Mh without serum (a, $P < 0.03$). Four days after inoculation, tracheal rings inoculated with Mh + CSS displayed greater vigor of ciliary beating than rings inoculated with Mh or Mh + NSS (b, $P < 0.004$). Results are means \pm SEM for 2 experiments.

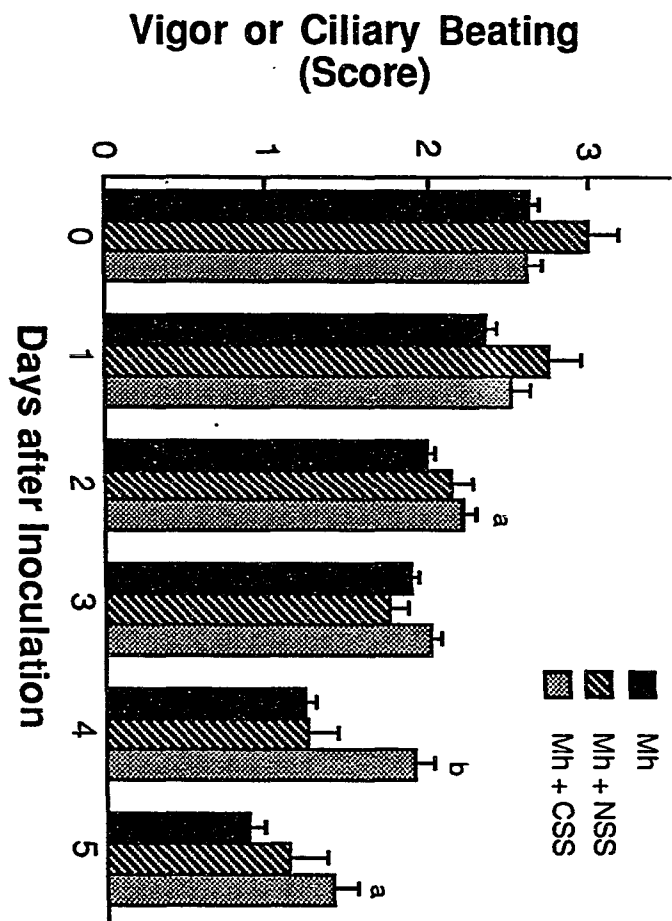


Figure 5. Ciliostasis induced by direct inoculation of porcine tracheal organ cultures with Mycoplasma hyopneumoniae (Direct), but not by indirect exposure through a 0.1 um membrane (Indirect). Each tracheal ring was placed on top of the 0.1 um membrane of a well insert. Mycoplasma hyopneumoniae was inoculated above and below (Direct) or only below (Indirect) the membrane. Five days after inoculation vigor of ciliary beating on tracheal rings separated from M. hyopneumoniae by the membrane was not significantly different from rings inoculated with sterile mycoplasma medium (Control). Significant ciliostasis of tracheal rings directly inoculated with M. hyopneumoniae was evident ($P < 0.014$). Results are means +/- SEM for 2 experiments.

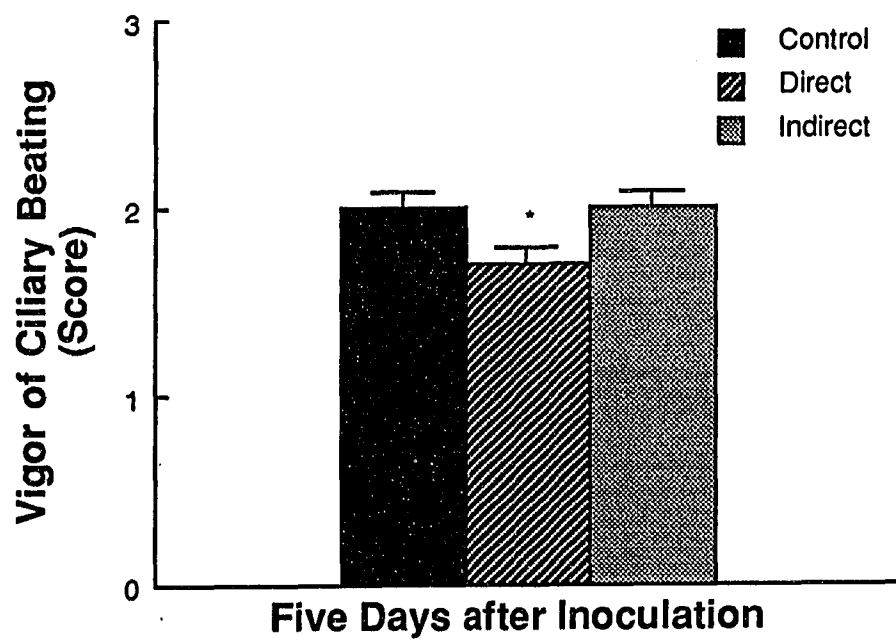


Figure 6. Loss of cilia from porcine tracheal ring epithelium after inoculation with dilutions of in vivo grown Mycoplasma hyopneumoniae. Five days after inoculation, rings inoculated with the 1:10 dilution exhibited more ciliary loss than all other dilutions (*, $P < 0.05$). Results are means \pm SEM for 3 experiments.

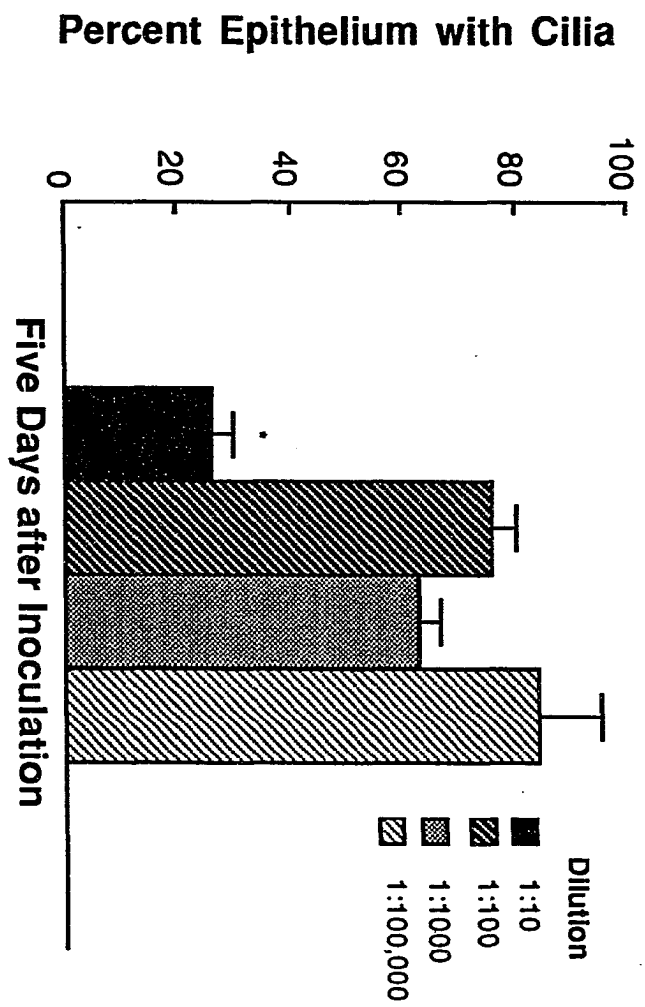


Figure 7.. Vigor of ciliary beating in porcine tracheal rings after inoculation with dilutions of in vivo grown Mycoplasma hyopneumoniae. Five days after inoculation, rings inoculated with the 1:10 dilution displayed significantly more ciliostasis than all other dilutions (*, $P < 0.005$). Results are means +/- SEM for 3 experiments.

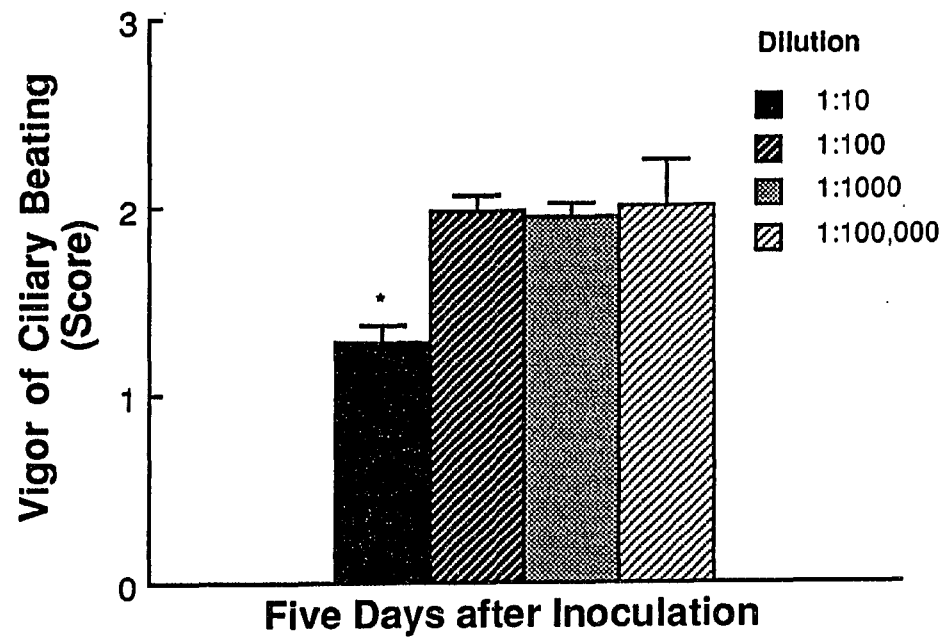


Figure 8. Dextran sulphate (DXS) inhibition of ciliary loss induced by Mycoplasma hyopneumoniae (MHP) in porcine tracheal rings. Five days after inoculation, tracheal rings inoculated with MHP displayed marked loss of cilia when compared to rings inoculated with sterile medium (Control) or MHP + DXS (*, $P < 0.05$). Results are means \pm SEM for 1 experiment (N = 10 tracheal rings for each data point).

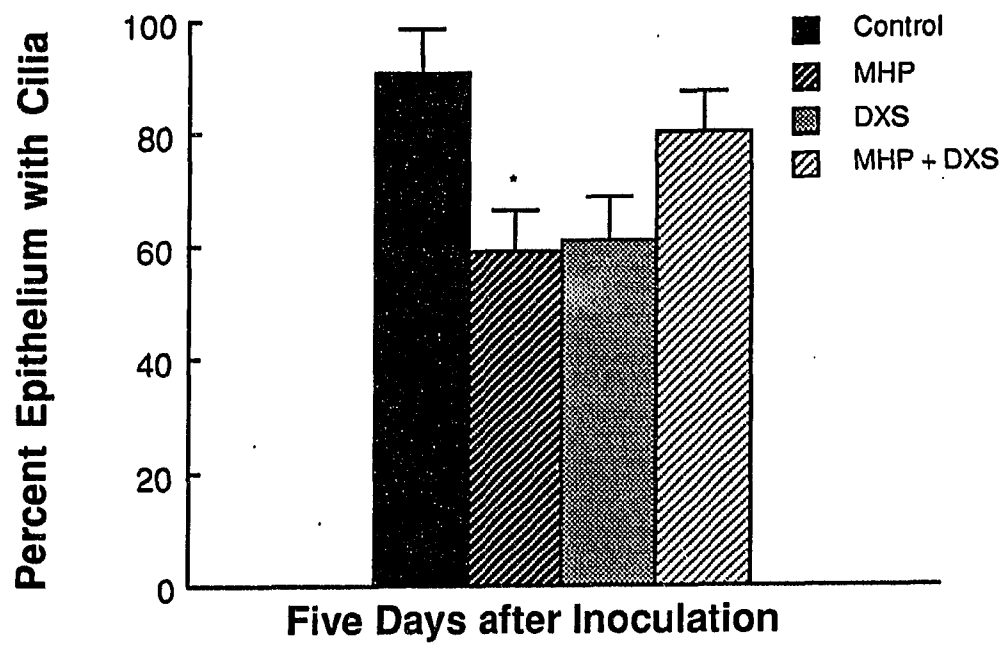


Figure 9. Dextran sulphate (DXS) inhibition of ciliostasis induced by Mycoplasma hyopneumoniae (MHP) in porcine tracheal rings. Five days after inoculation, tracheal rings inoculated with MHP displayed marked ciliostasis when compared to rings inoculated with sterile medium (Control), DXS or MHP + DXS (*, $P < 0.001$). Results are means \pm SEM for 1 experiment (N = 10 tracheal rings for each data point).

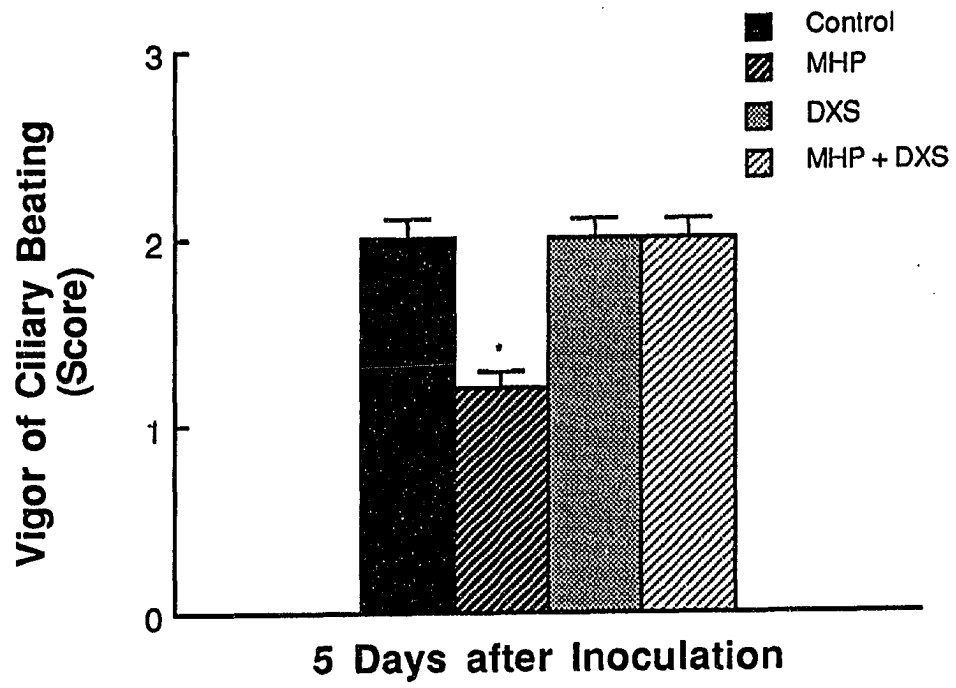


Figure 10. Percent epithelium with cilia 5 days after inoculation of porcine tracheal rings with Mycoplasma hyopneumoniae (MHP) grown with or without lung homogenate from a noninfected pig (NIP) or heated (100C) lung homogenate from a pig infected with M. hyopneumoniae (Heated Mh). Comparisons between passages are not possible, because each passage was a separate experiment. Growth of MHP in the presence of lung homogenate did not significantly enhance ability of either passage to induce loss of cilia. Results are means +/- SEM for 1 experiment using passage 5 (N = 8 tracheal rings for each data point), and 1 experiment using passage 10 (N = 13-15 tracheal rings for each data point).

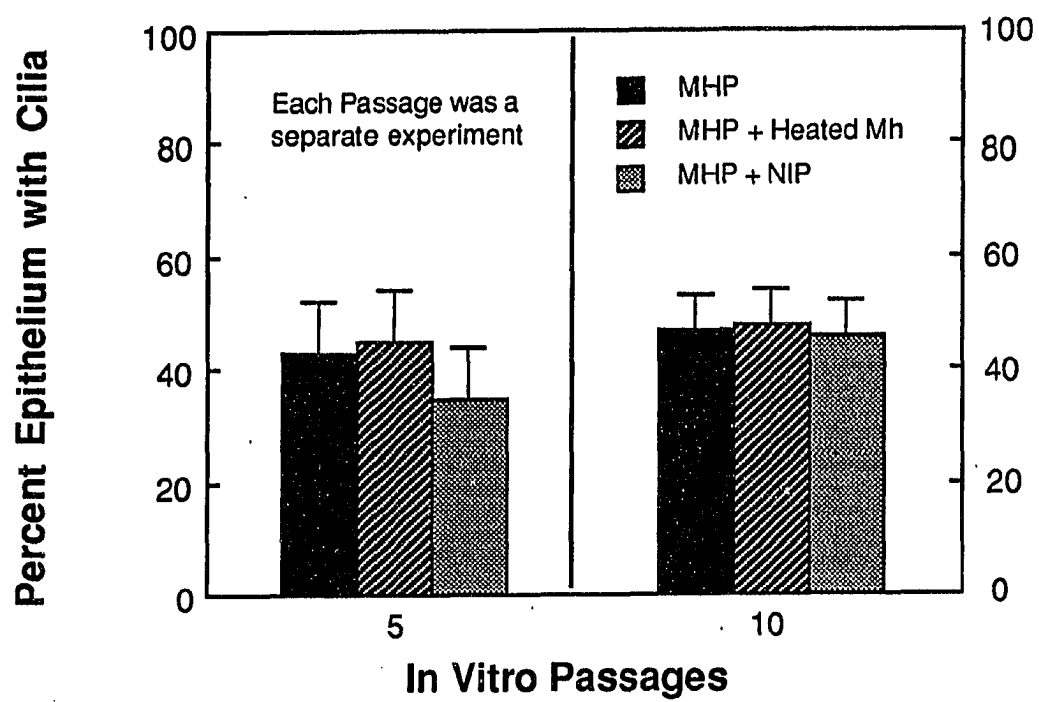


Figure 11. Vigor of ciliary beating 5 days after inoculation of porcine tracheal rings with Mycoplasma hyopneumoniae (MHP) grown with or without lung homogenate from a noninfected pig (NIP) or heated (100C) lung homogenate from a pig infected with M. hyopneumoniae (Heated Mh). Comparisons between passages are not possible, because each passage was a separate experiment. Growth of MHP in the presence of lung homogenate did not significantly enhance ability of either passage to induce ciliostasis. Results are means +/- SEM for 1 experiment using passage 5 (N = 8 tracheal rings for each data point), and 1 experiment using passage 10 (N = 13-15 tracheal rings for each data point).

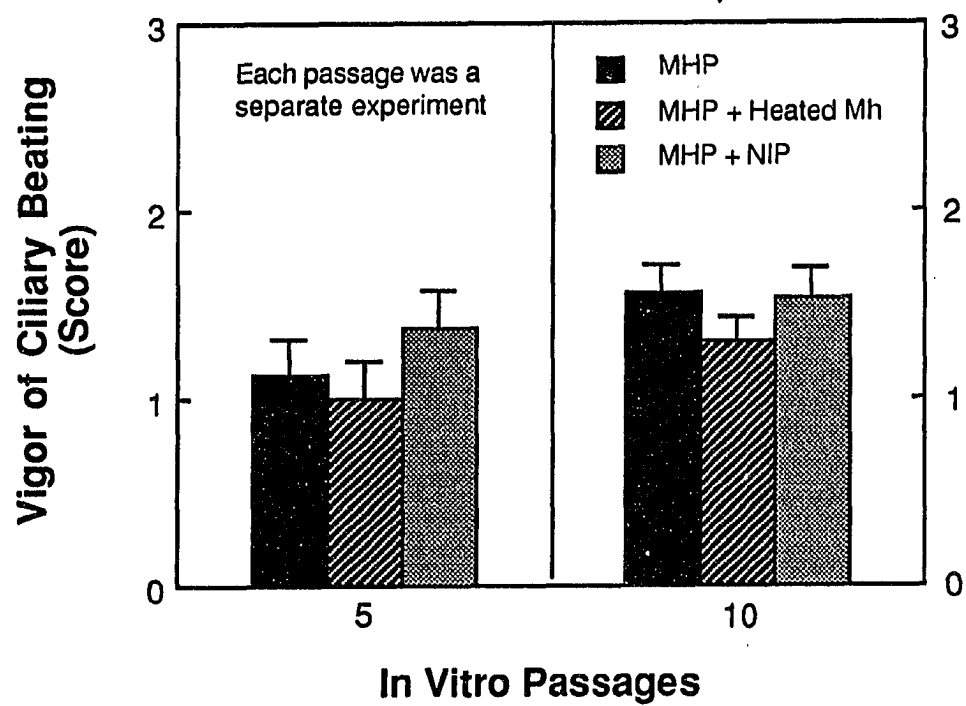


Figure 12. Inability of irradiated lung homogenate (Ir Mh) from a pig infected with Mycoplasma hyopneumoniae to induce cytotoxic ability in a noncytotoxic, highly passaged strain of M. hyopneumoniae (passage 40, strain 232, cloned; MHP-CL). After 5 days there was no significant difference in the retention of cilia by rings incubated with or without M. hyopneumoniae. Results are means +/- SEM for 1 experiment (N = 12-17 tracheal rings for each data point).

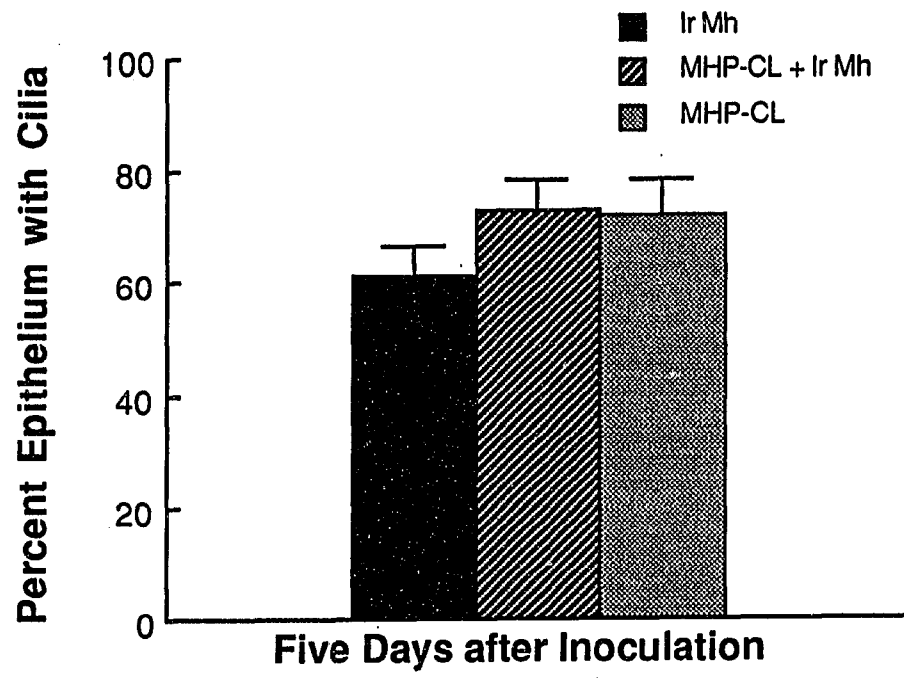


Figure 13. Inability of irradiated lung homogenate (Ir Mh) from a pig infected with Mycoplasma hyopneumoniae to induce ciliostatic potential in a nonciliostatic, highly passaged strain of M. hyopneumoniae (passage 40, strain 232, cloned; MHP-CL). After 5 days there was no significant difference in ciliostasis induced in rings incubated with or without M. hyopneumoniae. Results are means +/- SEM for 1 experiment (N = 12-17 tracheal rings for each data point).

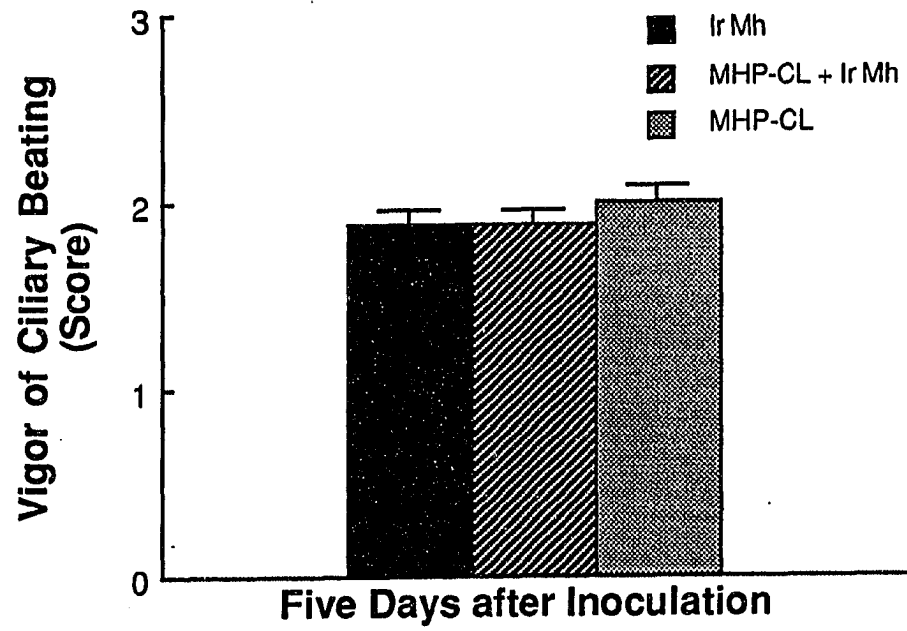


Figure 14. Porcine tracheal rings were inoculated with lung homogenate from a noninfected pig (NIP) without (Control) or with M. hyopneumoniae (passage 2; MHP). Some of the rings received NIP on day 0 only (NIP 1 day), and spent medium was replaced daily with sterile mycoplasma medium. Other rings received NIP on days 0-4, and spent medium which was removed daily was replaced with sterile mycoplasma medium containing NIP (NIP 5 days). Rings inoculated with MHP exhibited significant loss of cilia when compared to the corresponding 1 day (a, $p < 0.002$) or 5 day (b, $p < 0.0007$) NIP control. Results are means \pm SEM for 1 experiment (N = 16-17 tracheal rings for each data point).

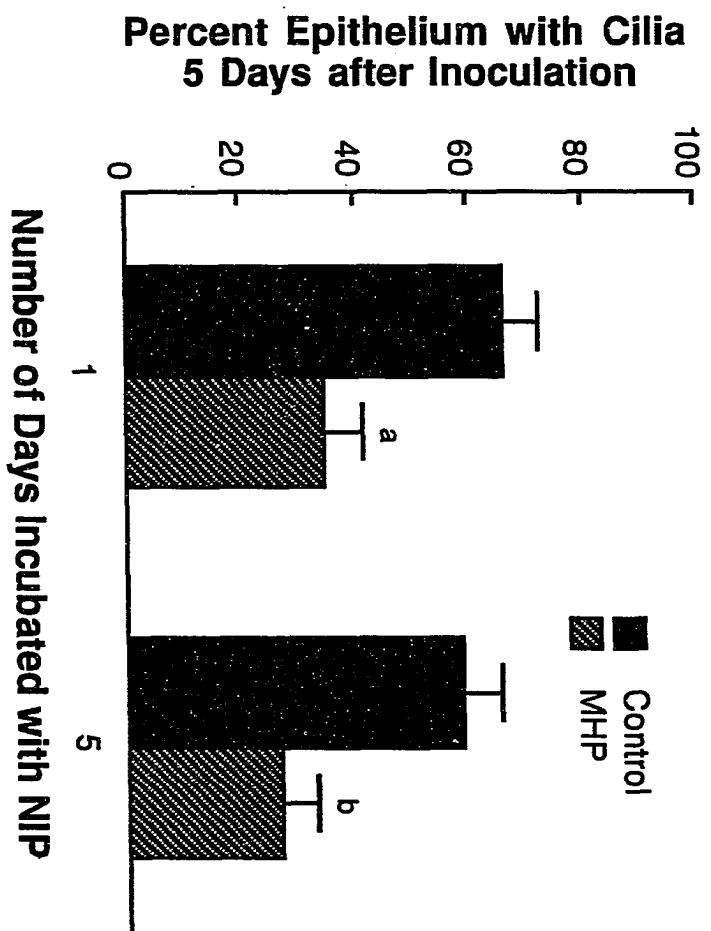


Figure 15. Porcine tracheal rings were inoculated with lung homogenate from a noninfected pig (NIP) without (Control) or with M. hyopneumoniae (passage 2; MHP). Some of the rings received NIP on day 0 only (NIP 1 day), and spent medium was replaced daily with sterile mycoplasma medium. Other rings received NIP on days 0-4, and spent medium which was removed daily was replaced with sterile mycoplasma medium containing NIP (NIP 5 days). Ciliostasis was more severe in rings receiving M. hyopneumoniae when compared to the corresponding 1 day (a, $p < 0.0001$) or 5 day (b, $p < 0.0001$) NIP control. Rings receiving MHP on day 0 and NIP for 5 days exhibited more loss of ciliary vigor than rings receiving MHP and NIP on day 0 only (b, $p < 0.012$). Results are means \pm SEM for 1 experiment (N = 16-17 tracheal rings for each data point).

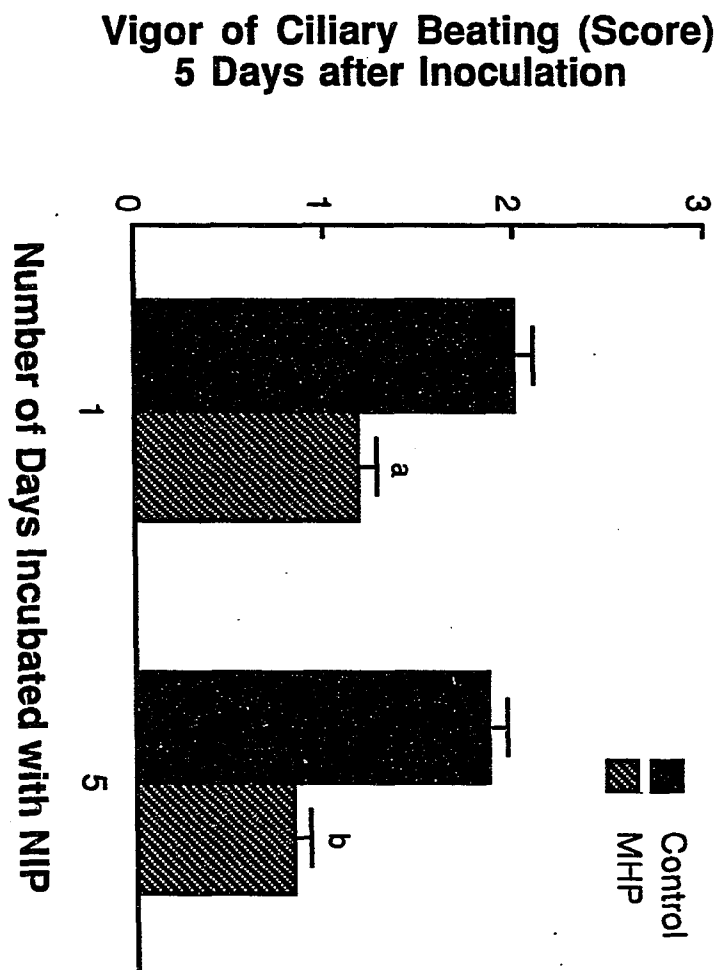


Figure 16. Percent epithelium with cilia after inoculation of porcine tracheal rings with porcine lung extract and Mycoplasma hyopneumoniae (MHP). Lung homogenate from a pig infected with M. hyopneumoniae was heated (100C) and centrifuged (740 x g) and the supernate was Heat Mh. Another aliquot of lung homogenate from a pig infected with M. hyopneumoniae was irradiated (180,000 rad) and centrifuged (740 x g) and the supernate was Cent Ir Mh. On day 0 tracheal rings were inoculated with lung homogenate from a noninfected pig (NIP), Cent Ir Mh, or Heat Mh without (Control) or with MHP. See page 86 for detailed methods of lung homogenate preparation. Tracheal rings inoculated with MHP with and without lung extract retained significantly less epithelium with cilia than controls receiving extract only (*, $p < 0.001$). Retention of ciliated epithelium was not significantly different between rings inoculated with MHP with or without lung extract. Results are means +/- SEM for 1 experiment (N = 6 tracheal rings for each data point of Heat LH; N = 9 tracheal rings for each data point of all other categories).

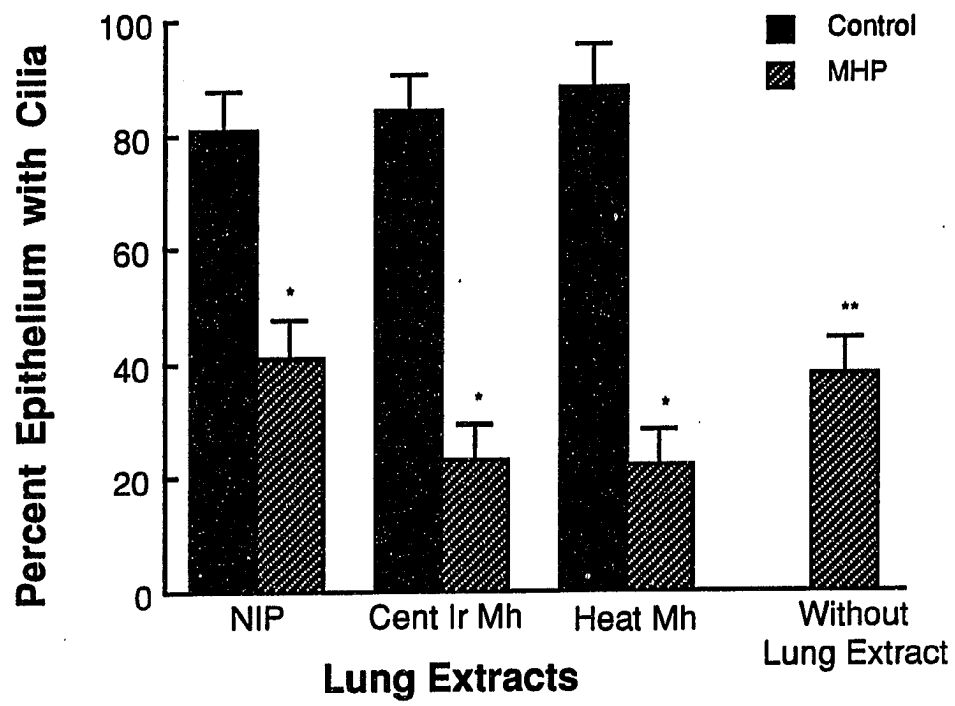


Figure 17. Loss of ciliary activity after inoculation of porcine tracheal rings with porcine lung extract and Mycoplasma hyopneumoniae. Lung homogenate from a pig infected with M. hyopneumoniae was heated (100C) and centrifuged (740 x g) and the supernate was Heat Mh. Another aliquot of lung homogenate from a pig infected with M. hyopneumoniae was irradiated (180,000 rad) and centrifuged (740 x g) and the supernate was Cent Ir Mh. On day 0 tracheal rings were inoculated with lung homogenate from a noninfected pig (NIP), Cent Ir Mh, or Heat Mh without (Control) or with MHP. See page 86 for detailed methods of lung homogenate preparation. Tracheal rings inoculated with MHP with and without lung extract exhibited significant ciliostasis when compared to controls receiving extract only (*, $p < 0.001$). Ciliary activity was not significantly different between rings inoculated with MHP with or without lung extract. Results are means +/- SEM for 1 experiment ((N = 6 tracheal rings for each data point of Heat LH; N = 9 tracheal rings for each data point of all other categories).

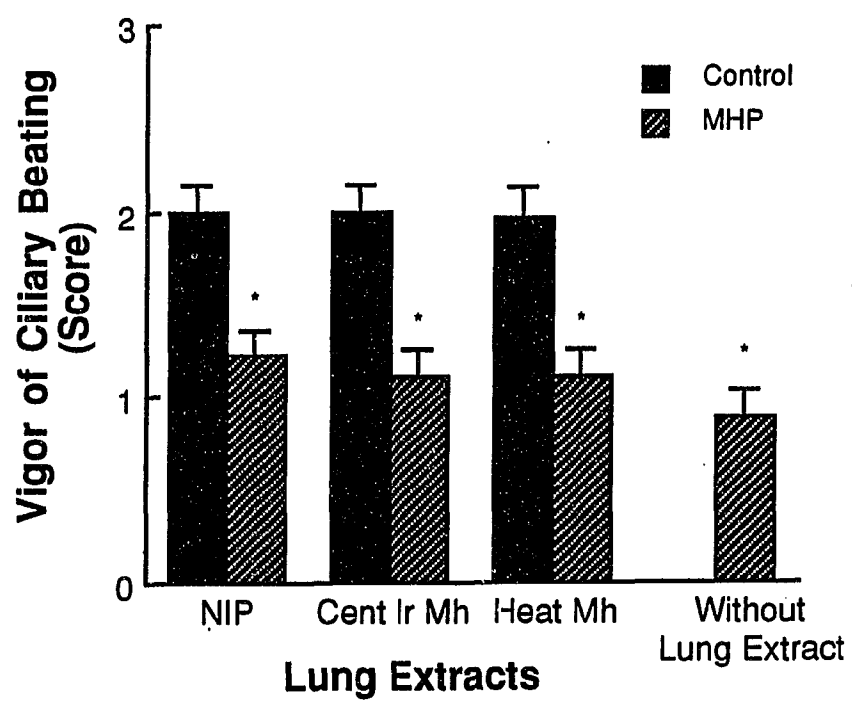


Figure 18. Loss of cilia by porcine tracheal rings after inoculation with Mycoplasma hyopneumoniae and/or lung preparations. Lung homogenate from a pig infected with M. hyopneumoniae was heated (100C), centrifuged (740g) and the supernate was passed through a filter retaining molecules greater than approximately 10,000 molecular weight (MW). The effluent, or filtrate (Fil; < 10,000 MW), and the retentate (Ret; > 10,000 MW) were inoculated to tracheal rings with or without M. hyopneumoniae (MHP). Loss of cilia was not significantly different for any of the preparations. Results are means +/- SEM for 1 experiment (N = 10-15 tracheal rings for each data point).

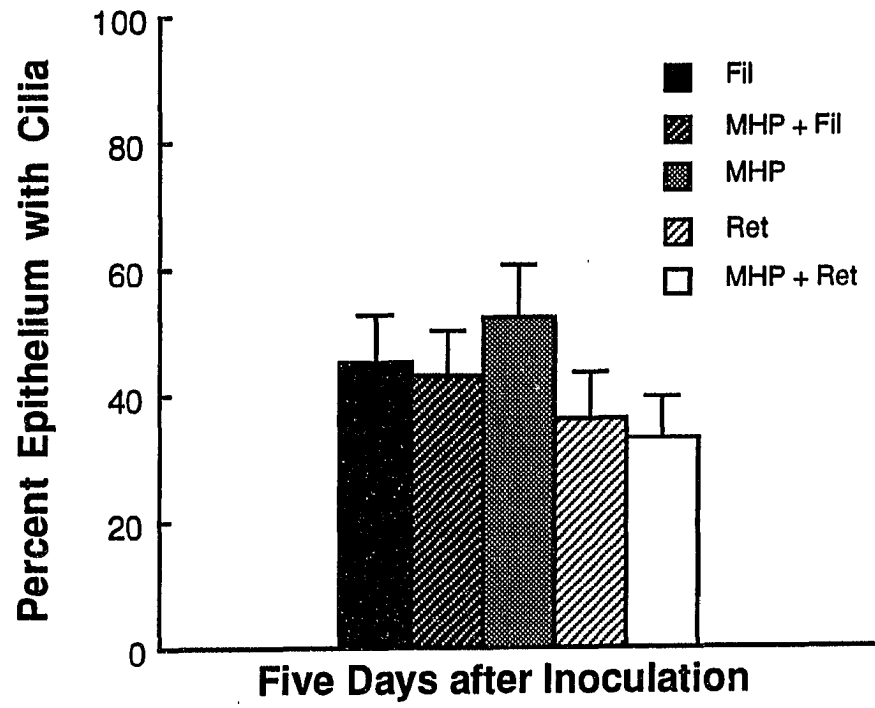


Figure 19. Loss of ciliary activity by porcine tracheal rings after inoculation with Mycoplasma hyopneumoniae and/or lung preparations. Lung homogenate from a pig infected with M. hyopneumoniae was heated (100C), centrifuged (740g) and the supernate was passed through a filter retaining molecules greater than approximately 10,000 molecular weight (MW). The effluent, or filtrate (Fil; < 10,000 MW), and the retentate (Ret; > 10,000 MW) were inoculated to tracheal rings with or without M. hyopneumoniae (MHP). Loss of ciliary activity was significantly greater in rings inoculated with Ret with or without MHP when compared to rings inoculated with Fil or MHP only (*, $p < 0.04$). Results are means \pm SEM for 1 experiment (N = 10-15 tracheal rings for each data point).

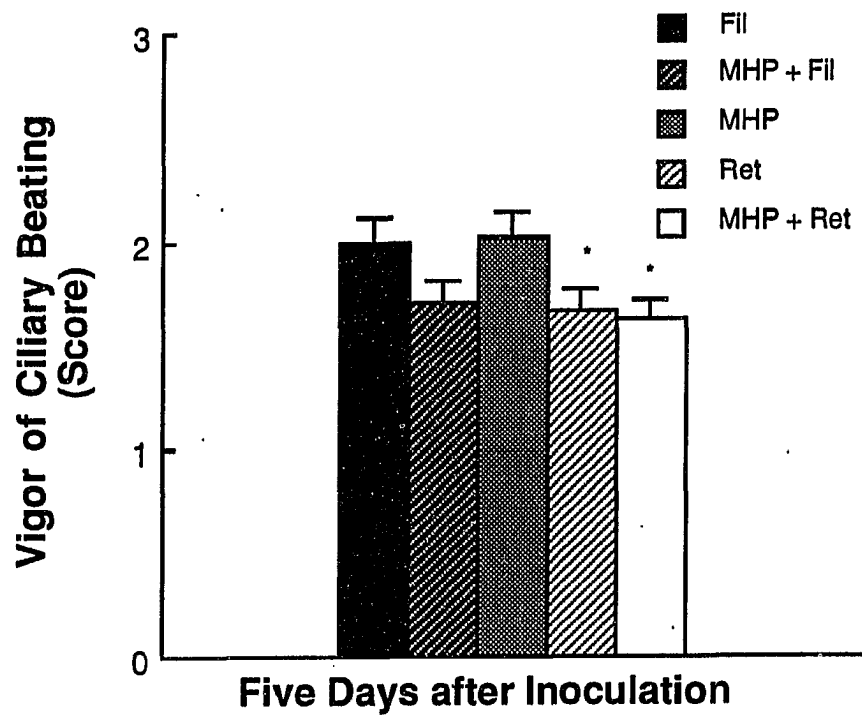


Figure 20. Dehydrogenase enzyme levels of porcine tracheal rings inoculated with lung homogenate from a pig infected with Mycoplasma hyopneumoniae (Mh), lung homogenate from a noninfected pig (NIP), passage 2 of Mh (MHP), or passage 2 of NIP (NIP-P). Five days after inoculation, presence of M. hyopneumoniae did not alter dehydrogenase enzyme activity in porcine tracheal rings. Results are means +/- SEM for 1 experiment (N = 11-12 tracheal rings for each data point).

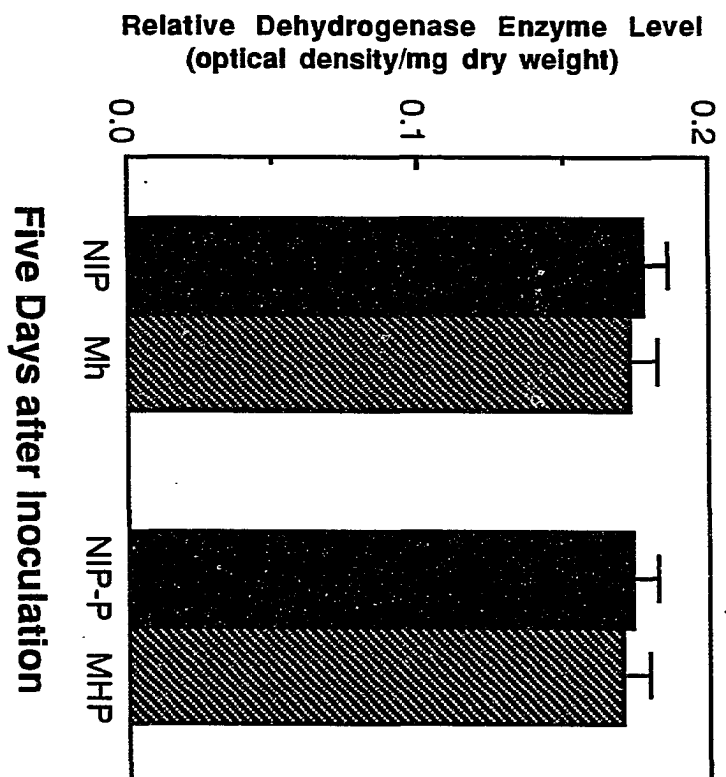


Figure 21. Intracellular calmodulin levels in porcine tracheal ring epithelium 2 and 5 days after inoculation with Mycoplasma hyopneumoniae (MHP). After incubation, the epithelium was removed from the cartilagenous portion of the tracheal explant and weighed to obtain a wet weight. The tissue was homogenized, and centrifuged before heating (100C) to obtain total (740g) or unbound (105,000g) calmodulin preparations. Total protein of the preparations was measured. Calmodulin content in ug was calculated with regard to mg wet weight (A) and mg protein (B) of the epithelium. No significant differences were detected between explants inoculated with MHP or uninoculated controls (Control). Results are means +/- SEM for 1 experiment (N = 5-6 tracheal rings for each data point at 2 days; N = 8-10 tracheal rings for each data point at 5 days).

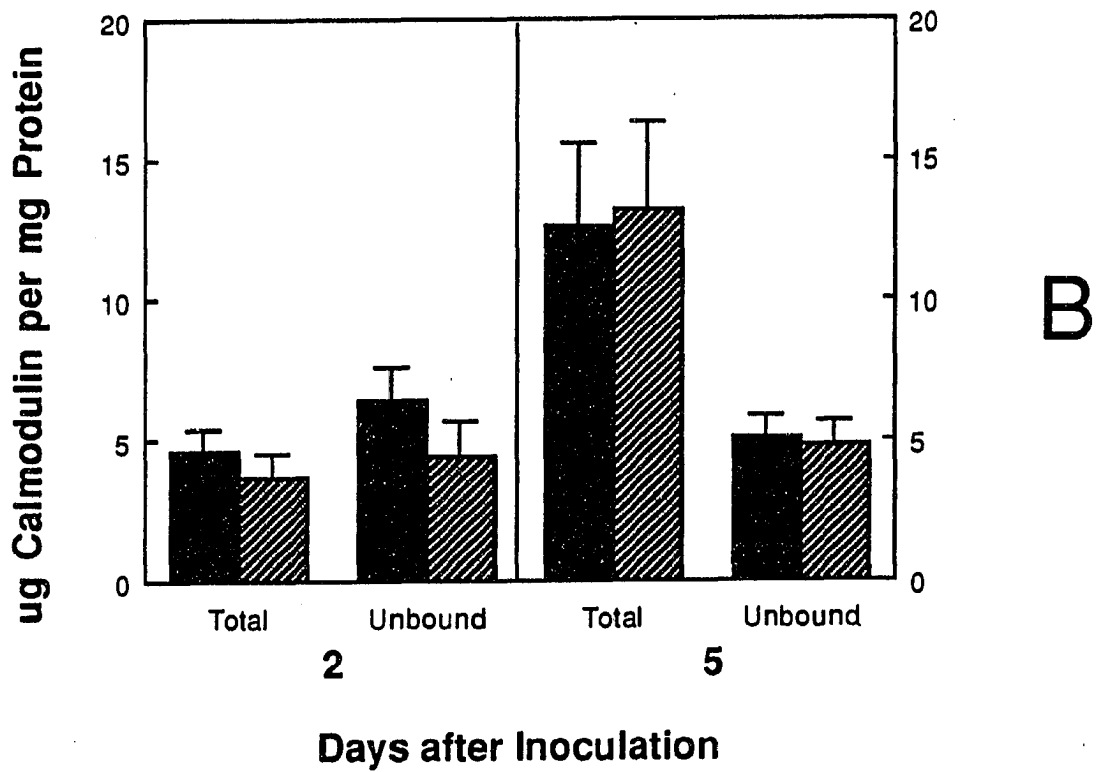
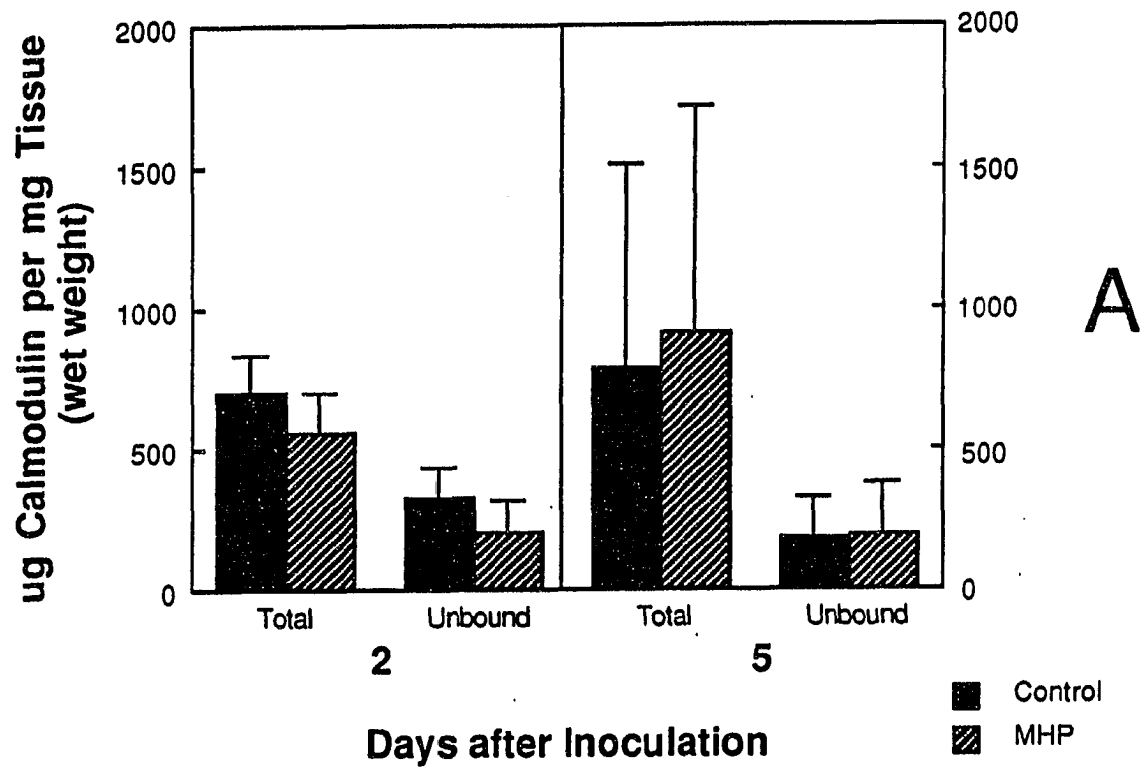


Figure 22. Extracellular calmodulin levels in the medium bathing porcine tracheal rings 2 and 5 days after inoculation with Mycoplasma hyopneumoniae (MHP). Volume of medium removed from each explant was measured daily and pooled for measurement of extracellular calmodulin. The medium was heated (100C), centrifuged (105,000g) and protein and calmodulin content were measured. Calmodulin content in ug was calculated with regard to total volume of medium (A) and mg protein (B) of the medium surrounding each explant. No significant differences were detected between explants inoculated with MHP or uninoculated controls (Control). Results are means +/- SEM for 1 experiment (N = 4-6 tracheal rings for each data point at 2 days; N = 3-10 tracheal rings for each data point at 5 days).

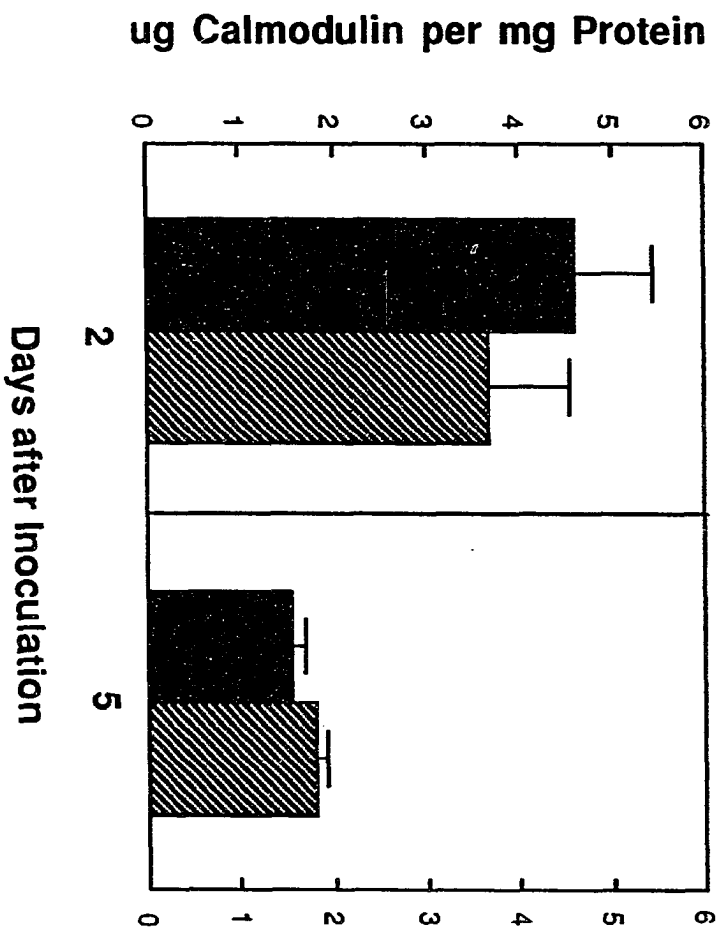
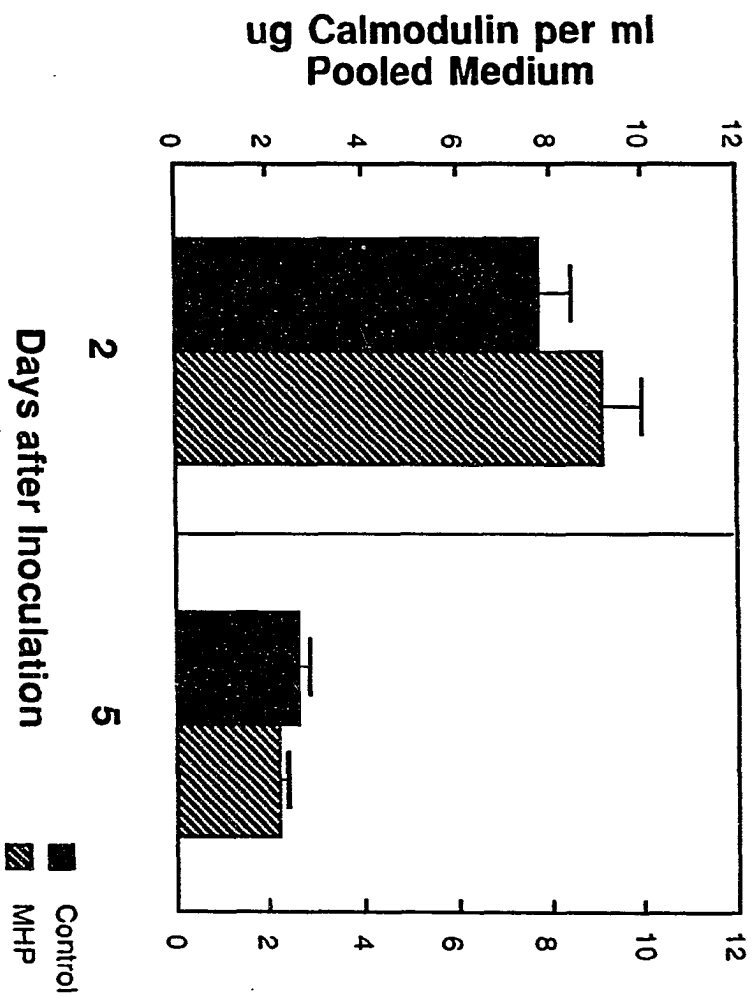
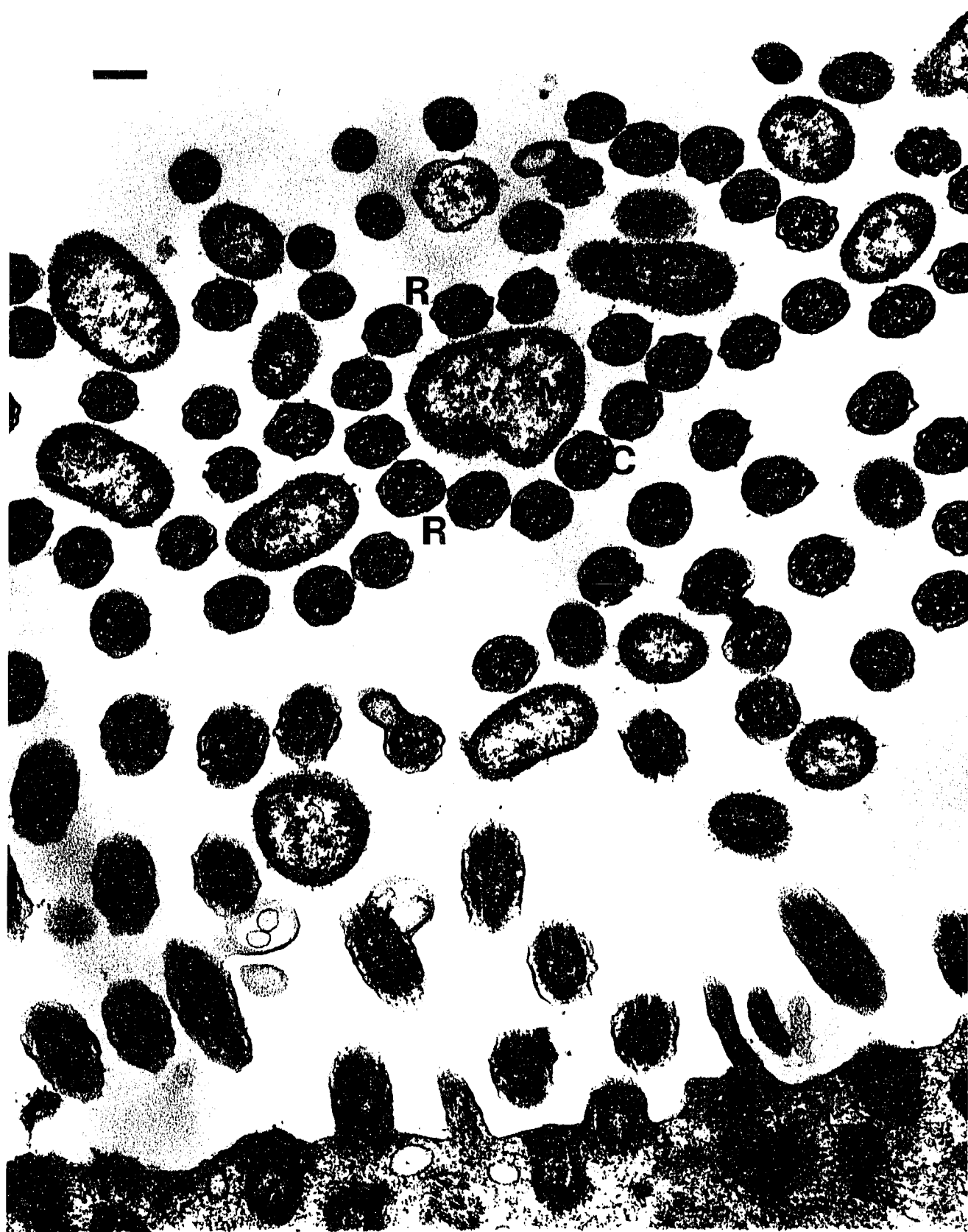


Figure 23. Electron micrograph of porcine tracheal ring ciliated epithelium 4 days after inoculation with in vivo grown Mycoplasma hyopneumoniae. Mycoplasmas (M) have hairlike fibrils extending from their surface to other mycoplasmas or to cilia (C). Cilia connected to mycoplasma by thin fibrils sometimes cluster around a single organism in a rosette-like pattern (R). Bar = 0.25 um.



APPENDIX B: EFFECTS OF NONCYTOTOXIC STRAINS OF MYCOPLASMA
HYOPNEUMONIAE ON PORCINE TRACHEAL ORGAN CULTURES

Porcine tracheal rings were inoculated with cultures of M. hyopneumoniae in an attempt to develop a tracheal organ culture model for study of pathogenesis and attachment.

MATERIALS AND METHODS

Media. Tracheal organ cultures were preincubated in MEM (Eagle's minimal essential medium with 2.2 mg/ml sodium bicarbonate, 2.0 mg/ml lactalbumin hydrosylate, 0.29 mg/ml L-glutamine, 2.0 mg/ml N-2-hydroxyethylpiperazine-N'-ethane sulfonic acid [HEPES], and 1000 U/ml potassium penicillin G). In 24 well tissue culture plates, the medium was MEM-S (MEM + 20% swine serum free of ELISA antibody to M. hyopneumoniae). The HEPES was omitted from the medium of one experiment.

Mycoplasma hyopneumoniae. Strain 144L (cloned, passages 19-23) was the pathogenic strain, and induced pneumonic lesions in pigs when inoculated intratracheally. The nonpathogenic strain J (ATCC 25934, passages 56-63) caused no lesions in pigs intratracheally inoculated with the organism. Each M. hyopneumoniae culture was diluted tenfold in Friis mycoplasma broth and incubated 24-48 hours to provide M. hyopneumoniae cultures.

Tracheal Rings. Crossbred Hampshire and Yorkshire piglets were obtained from sows from commercial swineherds by ceasarean section. Each piglet was deeply anesthetized and

the trachea aseptically removed at the level of the right bronchus. The excised trachea was cut into 20-25 rings. Ring 1 was adjacent to the bifurcation of the right bronchus, and rings 1-10 comprised the lower trachea for this experiment. Ring 20 or 25 was adjacent to the larynx, and rings 11-20 or 25 comprised the upper trachea for data collection. Each tracheal ring was placed in a tube with 1 ml MEM and rolled 1 revolution per minute at 37 C for 2-8 hours. Each ring was randomly assigned to a well in a 24 well tissue culture plate. Rings were incubated with or without M. hyopneumoniae in MEM-S at 37 C in 5.0% CO₂.

Experimental design. Control medium contained 1.0 part sterile Friis mycoplasma medium in 9 parts MEM-S. Medium with mycoplasmas contained 1 part M. hyopneumoniae culture in the log phase of growth and 9 parts MEM-S. Control or mycoplasma-containing MEM in 0.3 ml volumes was randomly assigned to each well of a 24 well tissue culture plate. Medium was not replenished. The medium from each well containing a ring was cultured for M. hyopneumoniae and other bacteria at the end of the experiment. In all experiments both strains of mycoplasma were diluted to 10⁻⁹, and all dilutions were inoculated onto tracheal rings.

Direct evaluation of damage to ciliated epithelium.

Ciliostasis and loss of cilia exhibited by tracheal ring epithelial cells was evaluated daily using an inverted

microscope at 100X. Percent epithelium retaining cilia (0-100%) was estimated and recorded. Ciliostasis, evaluated by observing the vigor of ciliary beating, was scored on a scale from 0 (no movement) to 3 (vigorous beating).

RESULTS

Cultures of M. hyopneumoniae did not induce ciliostasis or loss of cilia in this model. Percent epithelium with cilia was less for rings from the lower trachea than for rings from the upper trachea. However, rings and mycoplasmas were distributed randomly in each plate to prevent an effect on ciliary activity and retention data for all experiments in this thesis.

Information obtained during preliminary experiments demonstrated that less than 1000 U/ml potassium penicillin was not sufficient to significantly reduce contamination. The addition of fungicides to medium was discontinued during preliminary work because of damage inflicted to tracheal ring epithelium by these agents. Absence of HEPES from the medium did not affect ciliary activity or retention. Titration of the quantity of mycoplasma culture inoculated to each ring by diluting the mycoplasma culture to 10^{-9} did not reveal an optimum concentration of organisms (data not shown).

Data on ciliary activity and retention after inoculation with sterile medium or M. hyopneumoniae cultures are presented in Figures 1 and 2 of this appendix.

Figure 1. Effect of Mycoplasma hyopneumoniae on ciliated epithelium of porcine tracheal rings. Each ring was inoculated with sterile medium (Control), or a strain of M. hyopneumoniae pathogenic (144L) or nonpathogenic (J) for pigs. Retention of cilia (A) and ciliary activity (B) were evaluated daily starting the day after inoculation. Two days after inoculation tracheal rings receiving M. hyopneumoniae 144L retained a higher percentage of ciliated epithelium than controls, and the difference approached significance (a, $p < 0.0554$). Three days after inoculation of tracheal rings with 144L ciliary activity was significantly higher than in uninoculated controls (c, $p < 0.03$). Tracheal rings inoculated with J exhibited loss of cilia when compared to controls (b, $p < 0.009$), and decreased ciliary activity when compared to rings inoculated with 144L or uninoculated controls (d, $p < 0.0506$). However, cytotoxicity was likely induced by nutrient depletion rather than by a toxin, because replenishment of medium in subsequent experiments prevented the epithelial necrosis observed microscopically in these tracheal rings. Results are means \pm SEM for 5 experiments.

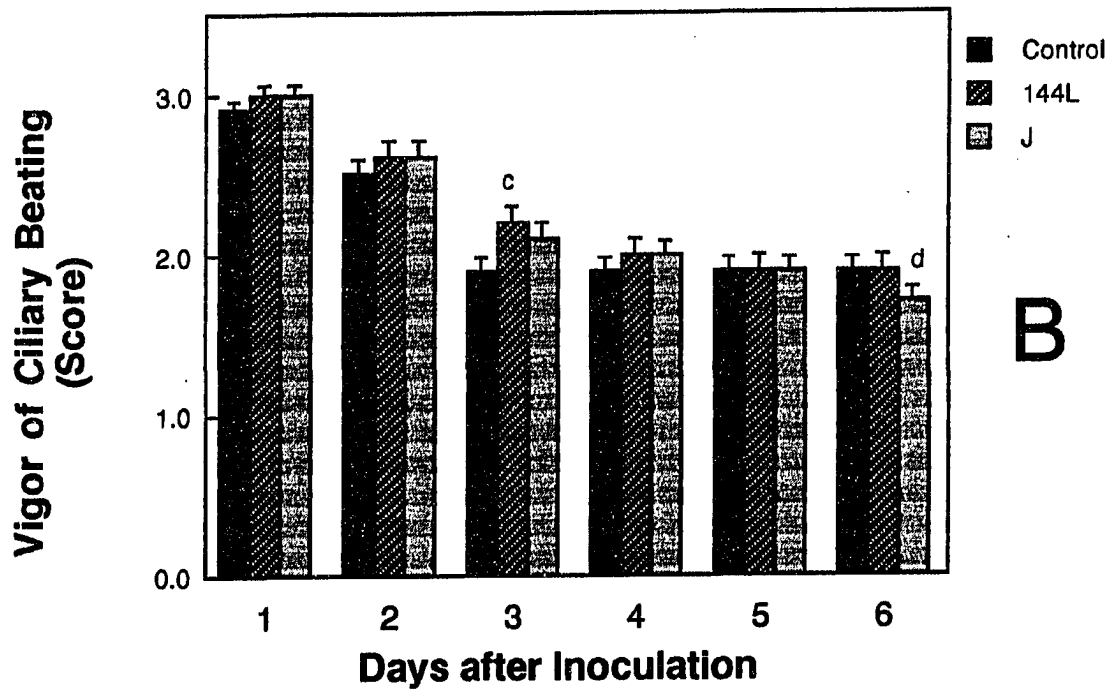
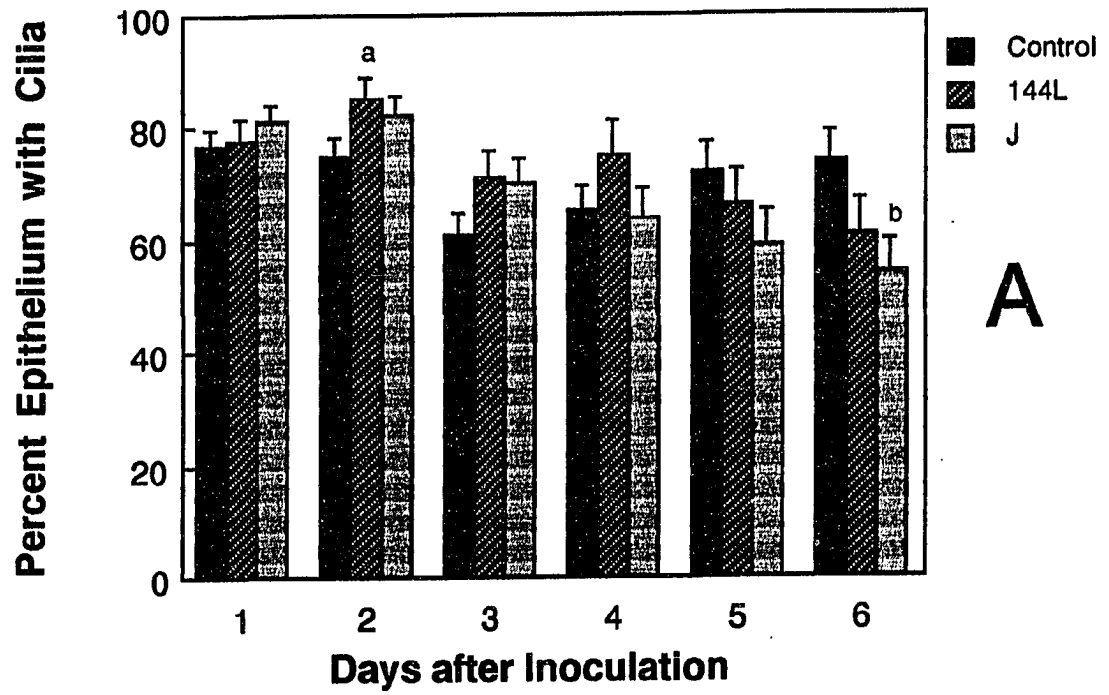
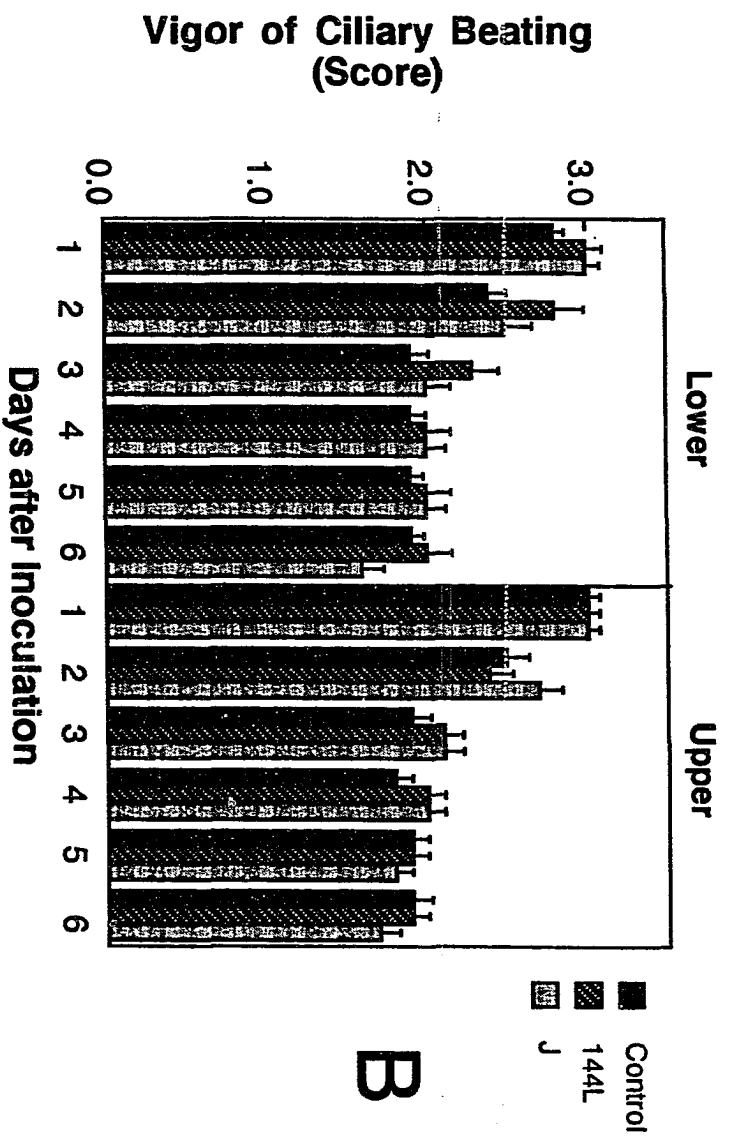
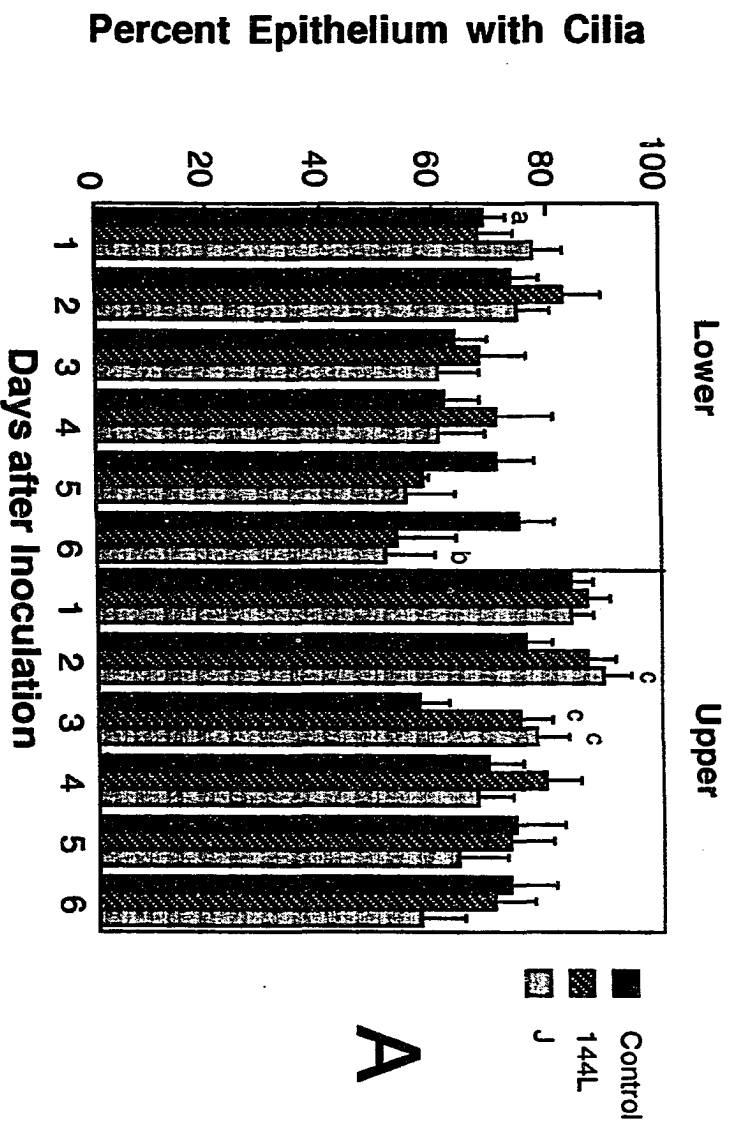


Figure 2. Effect of Mycoplasma hyopneumoniae on ciliated epithelium of porcine tracheal rings obtained from different locations of the trachea. Tracheal rings were assigned a number from 1 to 25. Ring 1 was adjacent to the bifurcation of the right bronchus and ring 20 or 25, adjacent to the larynx, was the last ring cut. Rings 1-10 were designated "lower", and rings 11-20 or 25 were designated "upper" for statistical analysis. Each ring was inoculated with sterile medium (Control), a pathogenic (144L), or a nonpathogenic (J) strain of M. hyopneumoniae. Retention of cilia (A) and ciliary activity (B) were evaluated daily starting the day after inoculation. After 1 day of incubation control rings obtained from the lower trachea exhibited significantly greater loss of cilia than control rings from the upper trachea (a, $p < 0.01$). Two days after inoculation tracheal rings receiving M. hyopneumoniae J retained a higher percentage of ciliated epithelium than controls ($p < 0.043$). Three days after inoculation tracheal rings receiving mycoplasma retained significantly more cilia than uninoculated controls (c, $p < 0.03$). After 6 days tracheal rings from the lower trachea which were inoculated with J exhibited significant loss of cilia when compared to lower trachea controls (b, $p < 0.029$). No differences in ciliary activity were detected at any time. Results are means \pm SEM of 5 experiments.



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